A STUDY OF THE BRUCELLA GROWTH-INHIBITING FACTOR IN BOVINE SERUM AND COLOSTRUM

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In view of the fact that previous studies had demonstrated a potentiating action of sulfadiazine on the specific brucella antibody-complement system and the role played by this combination of agents in infection (1), it seemed desirable to investigate in more detail the in vitro growth-inhibiting action of normal bovine serum and colostrum against Brucella with and without the presence of sodium sulfadiazine. The growth-inhibiting activity of normal serum and colostrum in the presence of sodium sulfadiazine was studied in the hope that it might provide a new avenue of approach to the problem of the resistance of calves to brucellosis and clarify the significance of the natural bactericidal antibody.

The resistance of calves up to 6 months of age and its lack of dependence on the presence of specific antibody (2) are well established facts. Inasmuch as accumulative evidence (3), (4), (5) indicates a relationship between the specific bactericidins and the resistance of cattle to brucellosis, investigators have sought to demonstrate a correlation between the natural bactericidins and the high resistance of calves. Irwin and Bell (6), who bred a strain of rabbits of high resistance to Brucella suis and another of low resistance, found that a correlation existed between the bactericidal activity of the blood and the resistance of the animal. Huddleson et al. (3) found no direct proof that the normal plasma bactericidins of calves accounted for their high resistance to brucella infection. However, these authors demonstrated that plasma from newborn calves showed little, or no, bactericidal action until after the ingestion

of colostrum and argued against ignoring the immunological significance of the natural antibody.

It is now well established that colostrum, the first milk after parturition, is the sole means of specific antibody transfer from the ruminant to its offspring (7). Within recent years Smith (8), (9) has isolated the component of colostrum associated with the passive transfer of specific antibodies to calves. This author (10) and Smith and Holm (11) have shown that this component and certain specific antibodies persist in the blood of the calf for many months. If one accepts the natural immunity phenomenon as analogous to that of the immune state, colostrum may be assumed to be the bearer of those substances imparting natural immunity to the calf. This is substantiated by the appearance of normal bactericidins in the blood of the calf after the ingestion of colostrum from normal cows. The investigation of natural colostral antibodies against Brucella appears to have been neglected.

Since a dairy herd known to have been free from brucellosis for more than 15 years was available, it afforded an opportunity to study serum and colostrum normal with respect to Brucella. In the present study the sulfadiazine-antibody-complement system has been used to demonstrate the presence of brucella growth-inhibition in colostrum and in the serum of calves at birth and to compare the growth-inhibiting activity of serum from newborn calves after the ingestion of colostrum with that of serum from adult cows.

Although the original purpose was to study the growth-inhibiting activity of serum from newborn calves and adult cows, certain observations on the effect of heat on this activity directed attention to characteristics of the normal growth-inhibiting factor which had not been reported

and to fundamental differences between the normal and specific growth-inhibitors. Fractions of specific and normal bovine serum were prepared and examined in an attempt to characterize the normal antibody as compared with the specific antibody.

In early experiments designed to study the bactericidal activity of plasma from animals having different statuses with respect to brucellosis, the brucella-infected animal was shown to be distinct in that its plasma failed to inhibit growth of Brucella (3). Huddleson (1) later found that this phenomenon was a manifestation of the well known prozone reaction and that prozone growth could be inhibited in vitro and in vivo in the guniea pig by the addition of fresh, normal rabbit serum and sodium sulfadiazine. In the following experiments normal bovine serum and colostrum have been shown to be capable of prozone-inhibition in the presence of sodium sulfadiazine, and the possible identity of this factor and the growth-inhibiting factor has been investigated.

GENERAL PROCEDURES

The method of Huddleson (1) for the *in vitro* estimation of growth-inhibiting action against *Brucella* was used throughout the experiments. With the exceptions noted in individual experiments the procedure was as follows:

Nature and source of blood specimens. -- Since it had been demonstrated that citrated plasma exerted a greater bactericidal action than serum and that the enhanced action was proportional to the amount of sodium citrate used as anticoagulant, serum was used in this study. However, some of the first specimens were plasma and certain plasma growth-inhibition tests contain comparative data that seem to permit their inclusion. All other samples were serums.

Blood specimens were collected from cows and calves normal with respect to infection with brucella organisms and from one brucella-infected cow. The normal animals were in a herd known to have been free from brucellosis for more than 15 years. None of the animals in this herd had ever been treated with vaccine. Colostrum was obtained from cows in the same herd.

Preparation of blood serum. -- The blood was collected aseptically and allowed to clot at room temperature. If the bactericidal test was carried out on the day of bleeding, unfiltered serum was used. Bovine serums to be stored and tested at intervals were filtered through a Hormann D8 pad in a Seitz filter and stored at 4° C. Normal rabbit serum collected on the day of use served as the source of added complement.

Preparation of colostral whey. -- Following the separation of fat and its removal, the colostrum was heated to 37° C. Sufficient commercial rennin to precipitate the casein within 1 hour was added and the

whole incubated for 1 hour at 37° C. The whey was sterilized by filtration through a Hormann D8 pad in a Seitz filter and stored at 4° C.

Preparation of sodium sulfadiazine. -- A solution containing 0.1 mg. of sodium sulfadiazine per ml. was prepared by adding 1.0 ml. of a 1.0-percent solution, which had been sterilized by filtration through a Hormann D6 pad in a Seitz filter, to 99 ml. of sterile distilled water. The solutions were prepared on the day of use.

Preparation of bacterial suspension. -- A smooth strain of Brucella abortus was used in all titration experiments. The organisms were grown on beef liver infusion agar slants for 24 hours at 37° C. The growth was removed, suspended in sterile dilution fluid, and the suspension standardized by a Libby photronreflectometer to a viable colony count of 1×10^6 per ml.

Titration of the growth-inhibiting action. -- All the titration experiments were made in liquid culture medium containing 1.5-percent Difco's "Bacto-Tryptose", 0.5-percent glucose, 0.5-percent sodium chloride, and 0.5 mg.-percent thiamine hydrochloride. The medium was filtered through paper and the pH adjusted to 6.7 with phosphoric acid. It was then dispensed in 5- or 10-ml. amounts and sterilized at 115° C. for 15 minutes.

The growth-inhibiting action of the sample was determined as follows: Serial twofold dilutions of the sample were made in 5 ml. of the culture medium. The other agents, 0.2 ml. fresh rabbit serum, 0.1 ml. of sodium sulfadiazine solution, and 0.1 ml. of the bacterial suspension (10^5 viable organisms), were added to each tube; the organisms were always added last. After thorough shaking, the cultures were incubated at 37° C. for 72 hours. The degree of visible growth was estimated at 24-hour intervals and recorded by the use of + and - signs. The highest serum dilution showing

no visible growth at 72 hours is referred to as the growth-inhibition titer.

EXPERIMENTS AND RESULTS

A. Study of the Agents Used for Determining the Brucella Growth-Inhibiting Action of Normal Bovine Serum and Colostrum

Experiments were conducted to examine (1) the efficacy of normal rabbit serum as a source of complement for measuring the growth-inhibiting activity of normal bovine serum in the presence of sodium sulfadiazine, (2) the effect of the growth-inhibitor in normal rabbit serum on the growth-inhibiting activity of normal bovine serum, and (3) the growth-inhibiting action of normal bovine serum and sodium sulfadiazine on different growth phases of Br. abortus. It was felt that the latter two effects might constitute significant sources of error in the determination of the activity of normal bovine serum, which is of relatively low order. In addition, while examining the reagents of the test, the mutual enhancement of the growth-inhibiting action of sodium sulfadiazine and normal bovine serum was demonstrated.

1. Normal rabbit serum as a source of complement

It is an established fact that complement is required in bactericidal systems, but the status of the interchangeability of complement from different species remains uncertain. Mackie and Finkelstein (12) found that absorbed serum from one species was capable of acting as complement with the natural bactericidal antibody from another species irrespective of the animal and type of typhoid-paratyphoid organism studied. Bovine and rabbit serums were among those apparently interchangeable. Irwin et al. (13) reported that unabsorbed guinea pig serum was unable to restore the bactericidal action of stored or heated bovine plasma against Br. abortus.

Pursuing this investigation, Shrigley and Irwin (14) showed that the absorbed serum complements from different animal sources were not always interchangeable in normal bactericidal action against Br. suis. In particular, rabbit complement activated only heated rabbit serum. Recently Coombs and Hale (15) have demonstrated the importance of the choice of complement when examining antiserums for the presence of complement-fixing antibodies.

Huddleson (1) found complement essential to the growth-inhibiting system acting in the presence of sodium sulfadiazine. In his work fresh, normal rabbit serum served as an adequate source of complement for the action of serum from brucella-infected guinea pigs, cows, and rabbits.

Before proceeding with this study it seemed desirable to demonstrate the efficacy of fresh, normal rabbit serum as a source of complement for the brucella growth-inhibitor of normal bovine serum in the presence of sodium sulfadiazine. The natural complement of three fresh bovine serums was inactivated by heating samples at 56° C. for 30 minutes. The activity of the heated serums was determined in the presence of sodium sulfadiazine both with and without fresh, normal rabbit serum. It may be seen from the data in table 1 that the heated serums without complement showed no growth-inhibiting action. However, the original activity was not reproduced upon the addition of rabbit complement. Sample 1, which originally possessed a prozone reaction, was less active. A prozone reaction and lowered activity appeared after heat inactivation of sample 2, and no activity was evidenced by the heated sample 3. These examples cited are representative of numerous similar results, many of which are included in the section on the effect of heat on the normal growth-inhibitor.

Since the modified action of heat-inactivated serum might result

Table 1

Effect of Fresh Normal Rabbit Serum on the Growth-Inhibiting Action of Heat-Inactivated Normal Bovine Serum

Serum No.	Serum	Agents added to serum dil.	Degree of growth, turbidity[a]					
			Dil. of serum, 1:					Bacterial control
			10	20	40	80	160	
1	Unheated	NaSD	3+	±	-	2+	4+	3+[b]
	Heated	NaSD	5+	4+	4+	4+	4+	3+[b]
	Heated	NRS and NaSD	5+	4+	2+	3+	4+	4+[c]
2	Unheated	NaSD	-	-	-	±	4+	4+[b]
	Heated	NaSD	4+	4+	4+	4+	4+	4+[b]
	Heated	NRS and NaSD	4+	-	-	4+	4+	4+[c]
3	Unheated	NaSD	-	-	1+	3+	4+	4+[b]
	Heated	NaSD	4+	4+	4+	4+	4+	4+[b]
	Heated	NRS and NaSD	4+	4+	4+	4+		4+[c]

[a] 10^6 Br. abortus added to each dilution and to controls (5 ml.).

[b] Control tube of medium also contains 0.01 mg. sodium sulfadiazine.

[c] Control tube of medium also contains 0.2 ml. of fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine.

NRS = 0.2 ml. of fresh, normal rabbit serum.

NaSD = 0.01 mg. sodium sulfadiazine.

Incubation period, 72 hours. Serum heated at 56° C. for 30 minutes.

- = no visible growth. + = degree of growth.

from thermolability of the antibody or inadequacy of the complement, the effect of fresh, normal rabbit serum on the growth-inhibitor of normal bovine serum and serum and plasma fractions which had been rendered complement-free by means other than heat was investigated. Serums 1 and 2 were stored at 4° C. for 5 and 6 weeks respectively. Fraction 3 was that portion precipitated from serum, as described elsewhere, by 20-percent sodium sulfate after removal of the material precipitated at 15-percent concentration of the salt. Two weeks intervened between bleeding of the animal and the determination of growth-inhibiting activity, and, in view of the manipulation in fractionation, dialysis, etc., it is unlikely that active complement remained. Fraction 4 was precipitated from serum by dialysis for 24 hours against a pH 5.4 phosphate buffer of 0.02 ionic strength. Although this method of isolation produces a minimum of protein denaturation, guinea pig and human complement are known to be inactivated by its use. The growth-inhibiting activity of this serum fraction was tested both 1 week and 8 weeks after bleeding of the animal. The data set forth in table 2 show that in the presence of sodium sulfadiazine fresh, normal rabbit serum functioned as complement with serums whose natural complement had deteriorated during storage and with fractions of normal serums devoid of complement as a result of the technique of preparation.

Certain dilutions of a 0.2-percent solution of Armour and Company's fraction III-1 from bovine plasma were found to inhibit growth in the presence of fresh, normal rabbit serum and sodium sulfadiazine. While it is highly improbable that such a pooled plasma represented only animals normal with respect to Brucella, the data in table 2 support the evidence that fresh, normal rabbit serum serves as an adequate source of complement for the sulfadiazine-potentiated action of the bovine growth-inhibitor

Table 2
Effect of Fresh Normal Rabbit Serum on the Growth-Inhibiting Action of Normal Bovine Serum, Serum Fractions, and a Plasma Fraction

Sample No.	Substance	Time between bleeding and testing (weeks)	Agents added to sample dil.	Degree of growth, turbidity[a]								
				Dilutions of sample, 1:								
				1.0×10^{1}	2.0×10^{1}	4.0×10^{1}	8.0×10^{1}	1.6×10^{2}	3.2×10^{2}	6.4×10^{2}	1.3×10^{3}	Bacterial control
1	Serum, whole	5	NaSD	5+	5+	4+	4+	4+				3+[b]
			NRS and NaSD	2+	-	1+	4+	4+				3+[c]
2	Serum, whole	6	NaSD	4+	4+	4+	4+	4+				4+[b]
			NRS and NaSD	2+	1+	2+	3+	4+				4+[c]
3	Serum, fraction: Pptd. at 20% Na_2SO_4	2	NaSD	4+	4+	4+	4+	4+				4+[b]
			NRS and NaSD	-	-	-	-	4+				4+[c]
4	Serum, fraction: Pptd. at pH 5.4-0.02μ	1	NaSD	4+	4+	4+						4+[b]
			NRS and NaSD	-	-	1+						4+[c]
4	Repeated	8	NaSD	4+	4+	4+						4+[b]
			NRS and NaSD	-	-	2+						4+[c]
5	Plasma, fraction: Armour's III-1 (0.2%)	-	NaSD	4+	4+	4+	4+	4+	4+	4+	4+	4+[b]
			NRS and NaSD	5+	4+	-	-	-	-	-	4+	4+[c]

[a] 10^{6} Br. abortus added to each dilution and to controls (5 ml.).
[b] Control tube of medium also contains 0.01 mg. sodium sulfadiazine.
[c] Control tube of medium also contains 0.2 ml. of fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine.
NRS = 0.2 ml. of fresh, normal rabbit serum. NaSD = 0.01 mg. sodium sulfadiazine.
Incubation period, 72 hours. - = no visible growth. + = degree of growth.

against Br. abortus.

2. The growth-inhibiting action of normal rabbit serum and its effect on the action of normal bovine serum

Since the agent, normal rabbit serum, used as a source of complement was known to possess slight bactericidal activity (1), (14), (16), the growth-inhibiting activity per se was investigated. In order to determine the activity, nine fresh, normal rabbit serums were examined on the day of bleeding, thus utilizing the complement of the serum sample instead of added complement. Although fresh, normal rabbit serum in the presence of sodium sulfadiazine proved capable of inhibiting growth of Br. abortus when diluted 1:5, and often at 1:10, in no instance did it show any activity at a dilution of 1:20 (table 3). That this action was not limited by dilution of the complement is evident from data offered elsewhere in which normal rabbit serum in a dilution of 1:25 invariably sufficed for added complement.

While normal rabbit serum was unable to inhibit growth in a dilution of 1:25, its concentration when used as a reagent in the test, the possibility of an additive action was investigated. The growth-inhibiting ability of nine fresh, normal bovine serums and one plasma was determined within 24 hours of bleeding. Five samples were filtered through Hormann D8 pads in Seitz filters. The growth-inhibiting activity of the five filtered and eight unfiltered samples in the presence of sodium sulfadiazine, both with and without fresh, normal rabbit serum, is shown in the data of table 4. Of the 13 samples tested, nine exhibited a slight increase in activity in the presence of normal rabbit serum, and four inhibited growth at the same dilution with and without normal rabbit serum. The effect was not apparent beyond one twofold dilution. It may be noted that serums 6 and 7 failed to inhibit growth completely in dilutions of

Table 3

Growth-Inhibiting Action of Fresh Normal Rabbit Serum

Rabbit No.	Degree of growth, turbidity[a]				
	Dilutions of serum, 1:				
	5	10	20	40	Bacterial control
1	-	4+	4+		3+
2	-	-	4+	4+	3+
3	-	1+	4+	4+	3+
4	-	-	4+	4+	3+
5	-	4+	4+	4+	4+
6	-	2+	4+	4+	4+
7	2+	2+	4+	4+	4+
8	-	-	4+	4+	4+
9	-	4+	4+	4+	4+

[a] 10^5 Br. abortus and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.
- = no visible growth. + = degree of growth.

Table 4

Effect of Fresh Normal Rabbit Serum on the Growth-Inhibiting Action of Normal Bovine Serum and Plasma

Sample No.	Agents added to sample dil.	Degree of growth, turbidity[a]						
		Dil. of sample, 1:						Bacterial control
		10	20	40	80	160	320	
1. Plasma, unfiltered	NaSD	-	-	-	2+	3+		3+[b]
	NRS + NaSD	-	-	-	-	3+		4+[c]
2. Serum, unfiltered	NaSD	-	-	-	-	3+	3+	3+[b]
	NRS + NaSD	-	-	-	-	±	4+	4+[c]
3. Serum, unfiltered	NaSD	-	-	-	C	1+	3+	3+[b]
	NRS + NaSD	-	C	-	-	-	3+	4+[c]
4. Serum, unfiltered	NaSD	-	-	-	-	-	4+	4+[b]
	NRS + NaSD	-	-	-	-	-	4+	4+[c]
4. Serum, filtered	NaSD	-	-	-	4+	4+	4+	4+[b]
	NRS + NaSD	-	-	-	-	4+	4+	4+[c]
5. Serum, unfiltered	NaSD	-	-	-	1+	4+	4+	4+[b]
	NRS + NaSD	-	-	-	-	4+	4+	4+[c]
5. Serum, filtered	NaSD	-	-	4+				4+[b]
	NRS + NaSD	-	-	-	4+			4+[c]
6. Serum, unfiltered	NaSD	-	-	-	1+	2+	2+	4+[b]
	NRS + NaSD	1+	1+	-	-	4+	4+	4+[c]
6. Serum, filtered	NaSD	-	-	2+	4+	4+	4+	4+[b]
	NRS + NaSD	1+	1+	-	3+	4+	4+	4+[c]
7. Serum, filtered	NaSD	-	-	-	4+	4+	4+	4+[b]
	NRS + NaSD	1+	1+	-	4+	4+	4+	4+[c]
8. Serum, filtered	NaSD	-	-	-	3+	4+	4+	4+[b]
	NRS + NaSD	-	-	-	4+	4+		4+[c]
9. Serum, unfiltered	NaSD	-	-	-	2+	4+	4+	4+[b]
	NRS + NaSD	-	-	±	±	5+	5+	4+[c]
10. Serum, unfiltered	NaSD	-	-	-	±	4+		4+[b]
	NRS + NaSD	-	-	-	-	3+	4+	4+[c]

[a] 10^6 Br. abortus added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Control tube of medium also contains 0.01 mg. sodium sulfadiazine.

[c] Control tube of medium also contains 0.2 ml. of fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine.

NRS = 0.2 ml. of fresh, normal rabbit serum.

NaSD = 0.01 mg. sodium sulfadiazine. C = contamination.

- = no visible growth. + = degree of growth.

1:10 and 1:20 when normal rabbit serum was present; no prozone growth was evident without it.

The normal rabbit serum used as a source of complement was incapable of growth-inhibiting action in the dilution at which it was used as a reagent in the sulfadiazine-antibody-complement test. However, it appeared to contribute slightly to the activity of normal bovine serum. Although the additive effect did not exceed one twofold dilution, which is within the error of the method, variations in the action of normal rabbit serum indicated that tests conducted with the same fresh rabbit serum would be more strictly comparable. Therefore, with the exception of samples tested after intervals of storage, all comparative data presented in the experiments were obtained from tests conducted with the same normal rabbit serum.

3. The growth-inhibiting action of normal bovine serum on five different growth phases of Br. abortus

Differences in sensitivity of diverse phases of organisms to various agents is generally recognized. Huddleson et al. (3) demonstrated that the plasma bactericidins affect smooth and rough phases of Br. abortus to a different extent and emphasized the importance of an undissociated cell suspension for testing growth-inhibition. The question arose as to whether other dissociated phases, in particular those not so readily distinguished from the smooth phase, might evidence differences in sensitivity and contribute errors to the results of the test.

Five growth phases of Br. abortus were compared as to sensitivity to the growth-inhibiting action of normal bovine serum in the presence of sodium sulfadiazine. Colonial characteristics of the phases were distinguished, in the manner described by Huddleson (17), by examination of colonies incubated for 4 days at 37° C. on tryptose agar medium using a

low power stereoscopic microscope with an oblique light source.

Of the five growth phases studied, the smooth-intermediate SI_1 most closely resembled the smooth S phase. Isolated SI_1 colonies could not be distinguished by sight from those of the S phase, but adjacent S and SI_1 colonies showed differences in opacity; the SI_1 colony was the more opaque. Colonies of the smooth-intermediate SI_2 phase had more opaque centers and cadmium yellow color than those of the S and SI_1 phases. Colonies of the mucoid M_1 phase appeared very similar to those of the S and SI_1 phases except for their slight mucoid consistency. The fifth phase studied was sector SI_1. Colonies of this growth phase were distinguished from the S phase by sectors extending from the centers to the borders of the colonies. Each succeeding transfer from a single sector colony on agar medium resulted in SI_1 and sector SI_1 colonies.

Data which demonstrate the comparative sensitivity of four growth phases of one Br. abortus strain to the growth-inhibiting action of a fresh and a stored serum are presented in table 5. The results typify those obtained with other serums. It may be noted that, in the presence of sodium sulfadiazine, growth of the M_1, S, SI_1 and sector SI_1 phase was inhibited by fresh serum 1 in dilutions of 1:10, 1:40, 1:40, and 1:80 respectively. Although the action of stored serum 2 was less pronounced, the same relative sensitivity of the four growth phases was apparent. Therefore, of the four phases, M_1 proved the least sensitive, S and SI_1 of equal sensitivity and exceeding that of M_1, and the sector phase the most sensitive to the growth-inhibiting action of normal bovine serum under the conditions of the test. An inspection of the results in the bacterial control tubes reveals that the relative degrees of sensitivity of these four phases of the same Br. abortus strain are reflected therein.

Table 5

Comparative Sensitivity of the Smooth Phase and Four Dissociated Phases of Brucella Abortus to the Growth-Inhibiting Action of Normal Bovine Serum

Serum No.	Phase	Degree of growth, turbidity[a]						
		Dilution of serum, 1:						Bacterial control
		10	20	40	80	160	320	
1	Smooth, S	-	-	-	5+	5+		5+
	Intermediate, SI_1	-	-	-	4+	5+	5+	5+
	Mucoid, M_1	-	2+	5+	6+	C	6+	6+
	Sector, SI_1	-	-	-	-	2+	3+	3+
2	Smooth, S	2+	2+	4+	4+	4+		4+
	Intermediate, SI_1	2+	3+	4+	4+	4+		4+
	Mucoid, M_1	4+	5+	5+	6+	6+		6+
	Sector, SI_1	-	-	1+	2+	2+	2+	3+
3	Smooth, S	-	-	-	3+	4+	4+	4+
	Intermediate, SI_2	-	-	-	-	-	1+	
4	Smooth, S	-	-	-	4+	4+	4+	3+
	Intermediate, SI_2	-	-	-	-	1+	1+	

[a] 10^6 Br. abortus, 0.2 ml. fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

C = contamination.

- = no visible growth. + = degree of growth.

The phases thus varied in sensitivity to normal bovine serum acting in the presence of sodium sulfadiazine and to sodium sulfadiazine alone.

The data also show a comparison of the sensitivity of an S and a smooth-intermediate SI_2 phase to the growth-inhibiting activity of two normal bovine serums. Growth of the SI_2 phase was inhibited by serums 3 and 4 in dilutions of 1:160 and 1:80 respectively; growth of the S phase was inhibited by these serums only in dilutions of 1:40 or less. The sensitivities of S and SI_2 phases from another strain were of the same relative order as those above. The results demonstrate that the SI_2 phase is more sensitive than the S phase and, accordingly, more than the SI_1 phase to the growth-inhibiting action of normal bovine serum.

Inspection of the data in table 5 reveals the wide range of bovine serum titers obtainable with bacterial suspensions of five pure growth phases. Differences that might be found in a series of tests due to changes in the growth phase of the organism used are readily appreciated. Not so readily known and considered is the similarity in the appearance of the smooth, mucoid M_1, and smooth-intermediate SI_1 phases (even SI_2 to the untrained observer). The necessity for constant vigilance to obviate such error is apparent.

4. Potentiating action of sodium sulfadiazine on the growth-inhibiting action of normal bovine serum and plasma

It has been shown by Huddleson (1) that certain concentrations of sodium sulfadiazine and specific anti-brucella serums which are ineffective by themselves exert growth-inhibiting action in a zone of dilutions when acting together. This author demonstrated that fresh, normal bovine serum enhanced the temporary growth-inhibiting effect of sulfonamides to a bactericidal effect.

In order to determine whether the presence of sodium sulfadiazine increased the activity of the growth-inhibitor in normal bovine serum against Br. abortus, the activity of serums and plasmas from animals of different age groups was determined with and without sodium sulfadiazine in the presence of fresh, normal rabbit serum. The results in table 6 are typical of all the samples tested. Both serum and plasma in the presence of complement alone proved unable to inhibit the growth of Br. abortus for 72 hours under the conditions of the test (several serums at low dilutions had shown a transient growth-inhibiting action at 24 hours). When sodium sulfadiazine plus complement were present, the serum or plasma of adult animals in dilutions of 1:40 and 1:80 inhibited visible growth. The sulfonamide showed a similar but less pronounced action with serum from newborn calves. That this effect was not due to the action of sodium sulfadiazine alone was evidenced by the growth of Br. abortus in its presence in the control tube. The foregoing results show that the growth-inhibiting action of normal bovine serum in the presence of sodium sulfadiazine represents a mutual enhancement of temporary growth-inhibiting properties inherent in the two substances.

Table 6

Potentiating Action of Sodium Sulfadiazine on the Growth-Inhibiting Action of Normal Bovine Serum and Plasma

Sample No.	Sample	Agents added to sample dil.	Degree of growth, turbidity[a]					
			Dilution of sample, 1:					Bacterial control
			10	20	40	80	160	
1	Serum, calf[d]	NRS	6+	6+	6+	6+	6+	6+[b]
		NRS + NaSD	-	-	3+	4+	4+	4+[c]
2	Serum, calf[d]	NRS	6+	6+	6+	6+	6+	6+[b]
		NRS + NaSD	2+	2+	4+	5+	5+	5+[c]
3	Plasma, heifer	NRS	6+	6+	6+	6+	6+	6+[b]
		NRS + NaSD	-	-	-	3+	3+	5+[c]
4	Plasma, cow	NRS	4+	5+	5+	5+	5+	6+[b]
		NRS + NaSD	-	-	-	-	3+	4+[c]
5	Serum, cow	NRS	5+	6+	6+	6+	6+	6+[b]
		NRS + NaSD	1+	1+	-	4+	4+	4+[c]
6	Serum, cow	NRS	6+	6+	6+	6+	6+	6+[b]
		NRS + NaSD	-	-	-	3+	4+	4+[c]
7	Serum, cow	NRS	4+	5+	6+	6+	6+	6+[b]
		NRS + NaSD	-	-	±	±	5+	4+[c]
8	Serum, cow	NRS	4+	5+	5+	5+	5+	6+[b]
		NRS + NaSD	-	-	-	-	3+	4+[c]

[a] 10^6 Br. abortus added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Control tube of medium also contains 0.2 ml. fresh, normal rabbit serum.

[c] Control tube of medium also contains 0.2 ml. fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine.

[d] Newborn calf, before colostrum.

NRS = 0.2 ml. of fresh, normal rabbit serum.

NaSD = 0.01 mg. sodium sulfadiazine.

- = no visible growth. + = degree of growth.

B. The Brucella Growth-Inhibiting Action of Normal Bovine Serum

1. Serum from cows

Many methods for estimating bactericidal activity against Brucella have been employed since Mackie and Finkelstein (16) demonstrated its presence in normal serum. These authors (12) devised an index method which involved the sterilization of a cell suspension using a constant amount of undiluted serum and decimal dilutions of a bacterial suspension. Using this method, Irwin and his associates (13) found that normal bovine serum destroyed 10^8 Br. abortus organisms per ml. in 24 hours. Irwin and Ferguson (4) extended the index method by adding complement to dilutions of serum in order to determine the highest dilution of serum capable of bactericidal action. Some activity was usually displayed by normal serum in a dilution of 1:25 and rarely in the next dilution, 1:125. Irwin and Beach (5) found that normal serum in the presence of added complement was active in dilutions of 1:40 or 1:80 and sometimes 1:160. The bactericidin in 0.3 ml. of undiluted serum from a normal cow killed 10^5 organisms of a virulent strain in 24 hours. When diluted 1:10, 1:20, 1:40 and 1:80, 0.3 ml. of the diluted serum killed 10^2, 10^3, 10^1, and 10^1 organisms respectively.

Huddleson and his associates (3) found that serums from 10 normal animals destroyed 10^5 to 10^6 Br. abortus organisms per ml. in 48 hours when the serum was in a dilution of 1:2 and various numbers of organisms were added. In establishing a procedure to reveal the maximum bactericidal activity of bovine serum or plasma, these authors, using both the number of organisms and amount of antibody as variables, came to the conclusion

that the antibody dilution method showed more distinct differences in bactericidal action. The discovery that sodium sulfadiazine enhanced the bactericidal action of serum led to the development of a method employing this salt (1). In this method, which proved far more sensitive for measuring the action of specific antibody than those cited, the antibody was diluted in liquid culture medium and a constant number of organisms added. The absence of, or suppression of, visible growth was termed growth-inhibiting action rather than bactericidal action.

In order to determine the brucella growth-inhibiting activity of serum from normal cows, fresh, unfiltered serums from 12 cows were examined. Three cows possessed a growth-inhibition titer of 1:160, eight of 1:80, and one of 1:40. Certain of these animals were bled on several occasions; the titer was reproducible in each case. Normal bovine serum in the presence of sodium sulfadiazine and added complement thus was capable of inhibiting the growth of 10^5 Br. abortus for 72 hours when in an average dilution of 1:80.

2. Serum from newborn calves

The high degree of resistance of young calves from normal dams to brucellosis led Huddleson and his associates (3) to investigate the bactericidal activity of blood from young calves as compared to that of the adult animal. It was observed that plasma from newborn calves exerted only slight, or no, killing action against a minimum number of organisms but that plasma from the same calves after the ingestion of colostrum killed large numbers of Br. abortus. However, the age at which calves showed maximum bactericidal action varied and the activity did not exceed that of the adult animal.

Although no correlation between demonstrable bactericidins and

resistance had been shown, the discovery of a more sensitive method for detecting the normal antibody prompted re-examination of the bactericidal activity of serum from calves. Many investigators (7) have found the transmission of specific antibodies to the calf to depend on the ingestion of colostrum during the first day of life and shown that specific antibodies reach a maximum concentration in the serum of the calf within 24 hours after the ingestion of colostrum. If the substance responsible for the protection of young calves against Brucella is transmitted by colostrum and demonstrable in the blood, it, too, should be present in maximum concentration immediately following the ingestion of colostrum.

To determine the growth-inhibiting activity in the presence of sodium sulfadiazine, serum samples were collected from four calves before and from three of the four calves after the ingestion of colostrum from their dams. The calves were allowed to remain with their dams from 24 to 48 hours after birth and had free access to colostrum during the time. An examination of the data in table 7 discloses, in the serums taken before the ingestion of colostrum, complete growth-inhibition at 72 hours by the serum of calf 1 in a dilution of 1:20 and partial inhibition by the serums of calves 2 and 3 in this dilution. The serum of calf 4, taken before colostrum but not shown in the comparisons of table 7, completely inhibited growth in a dilution of 1:20. Serum from calf 1 taken 24 hours after the ingestion of colostrum showed no significant increase in growth-inhibiting action. The activity of serums taken from calves 2 and 3 forty-eight hours after the ingestion of colostrum was definitely increased since they completely inhibited growth for 72 hours in dilutions of 1:40 and 1:20 respectively as compared to partial inhibition in dilutions of 1:20 at birth.

Table 7

Growth-Inhibiting Action of Serum from Newborn Calves Before and After the Ingestion of Colostrum

Calf No.	Ingestion of colostrum	Incubation period (hr.)	Degree of growth, turbidity[a]				
			Dil. of serum, 1:				Bacterial control
			10	20	40	80	
1	Before	48	-	-	1+	2+	3+
		72	-	-	3+	4+	4+
	24 hr. after	48	-	-	-	2+	3+
		72	-	-	2+	4+	4+
2	Before	48	-	-	1+	2+	4+
		72	2+	2+	4+	5+	5+
	48 hr. after	48	-	-	-	2+	2+
		72	-	-	-	4+	4+
3	Before	48	-	-	2+	3+	3+
		72	4+	2+	3+	4+	4+
	48 hr. after	48	-	-	-	2+	3+
		72	-	-	3+	4+	4+

[a] 10^8 Br. abortus, 0.2 ml. fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.).
- = no visible growth. + = degree of growth.

Although calves up to 6 months of age have been considered resistant to infection, this study did not reveal that their serums show a higher growth-inhibiting action against Brucella than do those from older animals. Serums taken from calves 48 hours after the ingestion of colostrum, although more active than before the ingestion of colostrum, were less active than serums from normal adult animals. In addition to confirming the results of Huddleson et al (3) as to the increase in, and relative concentration of, normal growth-inhibiting antibody after the ingestion of colostrum, the sulfadiazine-antibody-complement test also revealed that each calf possessed considerable growth-inhibiting activity prior to the ingestion of colostrum. Inasmuch as numerous workers (7) had failed to demonstrate specific antibodies in calf serum before the ingestion of colostrum, it was surprising to find growth-inhibiting antibody for Brucella in calf serum at birth.

C. The Brucella Growth-Inhibiting Action of Normal Bovine Colostrum

Appreciation of the significance of colostrum as a vehicle of antibody transfer to the young of certain species arose from the demonstration of specific antibodies in the colostrum and the appearance of these antibodies in the blood of the young only after the ingestion of colostrum. Presumably colostrum plays a similar role in the passage of natural antibodies. Although bactericidins have been shown to appear in the blood of the calf after the ingestion of colostrum from normal cows, no investigations of the normal brucella antibody of colostrum itself have been noted.

1. The growth-inhibiting action of colostrum

In order to determine the brucella growth-inhibiting activity of colostrum from normal cows in the presence of sodium sulfadiazine, colostrums from eight different animals were examined. All the colostrums were either the "first milking" or taken within one day post partum since this is the period when colostrum has been shown to be particularly high in antibody content.

In table 8 are shown the results of growth-inhibition determinations on the colostral wheys, fresh and heated at 56° C. for 30 minutes, with and without complement. The growth-inhibiting activity of the unheated colostrums, in the presence of sodium sulfadiazine and added complement, varied from slight inhibition in a dilution of 1:10 to complete inhibition in a dilution of 1:80. Seven of the eight colostrums possessed growth-inhibition titers, one of 1:10, two of 1:20, three of 1:40, and one of 1:80. Several colostrums exhibited partial growth-inhibition in higher dilutions.

Table 8

Growth-Inhibiting Action of Colostral Whey Irom No

Colostrum No.	Degree of growth, turbicity[a]															
	Unheated colostral whey										Heated colost					
	NaSD					NRS and NaSD					NaSD					
	Dilution of whey, 1:					Dilution of whey, 1:					Dilution of whey, 1:					D
	10	20	40	80	160	10	20	40	80	160	10	20	40	80	160	1
1	-	2+	4+	5+		-	-	2+	4+		-	4+				1
2	±	2+	4+	4+		-	-	-	4+	4+	4+	4+				1
3	-	-	2+	4+		-	-	-	-	2+	-	1+	4+			
4	-	2+	4+	4+		-	-	2+	2+	3+	1+	3+				1
5	1+	4+	4+	4+		1+	2+	4+	4+	4+	±	4+	4+	4+		1
6	-	3+	4+	4+		-	4+	4+	4+		-	4+	4+	4+		2
7	4+	4+	4+	4+		-	-	±	2+	4+	4+	4+	4+	4+		4
8	-	-	4+	4+		-	-	-	4+	4+	4+	4+	4+	4+		

[a]10^5 Br. abortus added to each dilution (5 ml.). Incubation period, 72
4+ growth.

[b]Whey heated at 56° C. for 30 minutes.

[c]Colostral whey added to a 1:100 dilution of serum from a brucella-infec
which also contains 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum,
NRS = 0.2 ml. fresh, normal rabbit serum added to each dilution.
NaSD = 0.01 mg. sodium sulfadiazine added to each dilution.
- = no visible growth. + = degree of growth.

Table 8

iting Action of Colostral Whey Irom Normal Cows

Degree of growth, turbicity[a]															
hey			Heated colostral whey[b]										Colostral whey added to test prozone growth inhibition[c] (ml.)		
and NaSD			NaSD					NRS and NaSD							
n of whey, 1:			Dilution of whey, 1:					Dilution of whey, 1:							
40	80	160	10	20	40	80	160	10	20	40	80	160	0.5	0.25	0.1
2+	4+		-	4+				1+	-	1+			2+	2+	
-	4+	4+	4+	4+				1+	±	2+			1+	1+	
-	-	2+	-	1+	4+			-	-	-	-	3+	-	1+	2+
2+	2+	3+	1+	3+				1+	-	2+	3+	4+	1+	3+	
4+	4+	4+	±	4+	4+	4+		1+	4+	4+	4+	4+	1+	2+	2+
4+	4+		-	4+	4+	4+		2+	4+	4+	4+	4+	1+	-	-
±	2+	4+	4+	4+	4+	4+		4+	3+	2+	3+	4+			
-	4+	4+	4+	4+	4+	4+		-	-	3+	4+	4+	2+	3+	4+

.ution (5 ml.). Incubation period, 72 hours. Bacterial controls,

ıtes.
lilution of serum from a brucella-infected cow in 5 ml. of culture medium 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine.
; serum added to each dilution.
ıe added to each dilution.
) of growth.

The colostrums thus possessed a definitely lower growth-inhibiting activity in the presence of sodium sulfadiazine and complement than did blood serums, which had an average titer of 1:80. In contrast to the results with this normal growth-inhibitor, it is a well established fact that the specific antibodies of colostrum generally exceed their concentration in the blood serum.

2. A colostral growth-inhibiting action independent of complement

In the course of demonstrating the effect of added fresh, normal rabbit serum alone and sodium sulfadiazine alone on colostral growth-inhibiting activity, an apparent departure from the classical bactericidal behavior of serum was noted. The colostrum alone exerted no activity under the conditions of this test nor did the colostrum plus fresh, normal rabbit serum as a source of complement. However, colostrum in the presence of sodium sulfadiazine did evidence activity. An examination of table 8 discloses that two wheys inhibited growth at a 1:20 dilution and four at a 1:10 dilution while one was capable of retarding growth and one showed no activity at a 1:10 dilution when tested with sodium sulfadiazine but without fresh, normal rabbit serum.

Since milk had been reported devoid of complement, no activity was anticipated in fresh, colostral wheys without the presence of added complement. Inasmuch as complement had recently been detected in fluids previously considered lacking in this complex, the colostrums were heated at 56° C. for 30 minutes to render any complement present inactive, and they were again tested for activity with sodium sulfadiazine. As may be observed in the data presented in table 8, the activity of samples 2 and 8 disappeared entirely on heating, but five samples, in the presence of sodium sulfadiazine alone, exhibited growth-inhibition although the

activity of four had been somewhat reduced. Therefore, some colostrums appeared capable of inhibiting growth without the presence of complement when sodium sulfadiazine was present. No analogous growth-inhibiting behavior was noted in blood serum.

If eight colostrums may be considered representative, the lack of correlation between the complement-free growth-inhibiting action and the growth-inhibiting action in the presence of complement suggests two separate growth-inhibiting entities in colostrum neither of which is entirely destroyed at 56° C. for 30 minutes. For example, the titer of unheated colostrums 5 and 6 remained unaltered by the addition of complement, seemingly indicating an antibody not requiring or enhanced by complement. On the other hand, sample 7 possessed no activity with sodium sulfadiazine alone but was found to be active in a dilution of 1:40 in the presence of complement and the salt. The growth-inhibiting activity of the remaining colostrums was apparent without complement and enhanced by its presence, more so than by any additive effect demonstrated with rabbit serum.

From an extensive study of the bactericidal reactions of normal serum, Mackie and Finkelstein (16) concluded that the thermostable principle of serum confined itself to activity only against the Gram-positive organisms whereas the thermolabile principle (so defined because of the complement requirement of the system) reacted against only the Gram-negative organisms. Previous workers had drawn attention to the existence of two different bactericidal mechanisms in normal serum but did not make this sharp differentiation as to Gram reaction. Fleming (18) found that the lysozyme of body tissues and secretions lysed both Gram-positive and Gram-negative organisms. He was able to show some lysis of Br. abortus but not of Br. melitensis. No attempt has been made here to characterize

the growth-inhibiting principle in colostrum which acts without complement, but its presence is notable.

D. The Brucella Growth-Inhibiting Factor of Bovine Serum Fractions Prepared by Sodium Sulfate Precipitation

No attempts to fractionate serum in order to study the natural bactericidal properties of the components appear to have been undertaken. While the isolation and purification of a single component from a system as complex as serum constitutes a major problem, it was felt that certain rudimentary information as to the nature of the growth-inhibiting antibody might be obtained by an examination of serum fractions.

Antibodies engendered by the injection of antigens have been shown to be associated with serum globulins and it is to be expected that normal antibodies might also prove to be of globulin nature. San Clemente and Huddleson (19) demonstrated by adsorption of specific antiserum that the brucella agglutinin of bovine serum was associated with the γ-globulin. Hess and Deutsch (20) also found specific brucella antibodies in bovine serum associated with the γ_1- and γ_2-globulins with a predominance in γ_1-globulin. The antibody activity of bovine antiserums against several other antigens was found in the T- and γ-components by Smith (8). That serum component migrating between the β- and γ-globulins has been variously termed T, β_2 and γ_1.

Limited data concerning the globulin nature of some natural antibodies resulted from studies of natural agglutinins and complement-fixing antibodies (12), (21), (22), (23), (24). These authors examined the activity of fractions prepared by carbon dioxide precipitation of euglobulin from a dilute serum solution. In certain species the euglobulin alone contained the antibody while serum from other species yielded fractions of equal and unequal activity.

In this experiment a modification of the Howe (25) sodium sulfate method was employed to separate three fractions from serum. Several considerations led to modification of the Howe method. Precipitation from serum in a dilution of 1:2 to 1:3, instead of Howe's 1:20, was based on the observation of Cohn and his associates (26) that this allowed effective precipitation of serum fractions by ammonium sulfate. Furthermore, successful precipitation of specific brucella antibody by ammonium sulfate had been effected at a serum dilution of 1:2 (19). It seemed advisable to precipitate the globulin at room temperature, instead of 37° C., as the thermolability of the normal growth-inhibitor was apparent by that time. The salt concentrations established as optimum in the Howe method were not strictly applicable to these preparations in view of the variations used in dilution and temperature (25).

It seemed possible that the natural growth-inhibitor might precipitate with the same fraction as the specific antibody and that a method which satisfactorily separated the more readily detected specific antibody might serve to fractionate the natural one. On the other hand, failure of the natural and specific antibodies to precipitate under the same conditions would indicate dissimilar properties of the two. With this goal, the salting-out of serum from a brucella-infected cow was studied, and the fractions obtained from normal and specific serums were examined and compared.

1. Fractionation of specific antiserum

a. Preparation of fractions. -- Serum 991, which possessed unusually high growth-inhibition and agglutination titers, was obtained from a brucella-infected cow. This serum was separated into three fractions. One volume of serum was diluted with one volume of 25-percent sodium

sulfate solution (at 37° C.) and allowed to stand at room temperature overnight. The resultant precipitate, termed the 12.5-percent fraction, was centrifuged, washed once with 12.5-percent sodium sulfate solution, and made up to one-half the original volume with water. Following dialysis for 3 days against 0.85-percent sodium chloride solution at 4° C., the solution was sterilized by filtration through a Hormann D8 pad in a Seitz filter and stored at 4° C. After removal of the 12.5-percent precipitate, sufficient dry sodium sulfate to bring the concentration of the supernatant to 15 percent was added slowly with mechanical stirring. After standing at room temperature overnight, the precipitate was washed once with 15-percent sodium sulfate solution, made up to one-third the original volume, dialyzed, and sterilized. The second fraction was designated as the 15-percent one. The supernatant obtained from precipitation at 15-percent salt concentration was dialyzed against running tap water for 40 hours, concentrated to original volume by evaporation for 10 hours at 37° C., dialyzed against 0.85-percent sodium chloride solution at 4° C. for an additional 2 days, and sterilized. After restoring the concentrated fractions to their original serum volume, the growth-inhibiting activity was determined in the customary manner. Nitrogen was determined by the Kjeldahl method.

When analyses revealed appreciable activity both in the 12.5- and 15-percent fractions, a single precipitation of serum 991 was made at 15-percent salt concentration to determine whether it would effect as complete a separation of the specific antibody as the two separate precipitations. A second single precipitation of this serum was made under slightly different conditions. It was thought that precipitation in the presence of normal serum would provide a more normal albumin-globulin ratio and approximate the conditions encountered in normal serum

fractionation. One part of serum 991 was diluted with two parts of normal bovine serum and precipitated at 15-percent concentration of sodium sulfate. Otherwise the procedure was as previously outlined. As determined from the growth-inhibiting activity, in both instances the same percent of total brucella growth-inhibiting antibody was precipitated by one precipitation as by the sum of the two separate precipitations.

b. Growth-inhibiting action. -- Serum 991 and its 12.5-percent fraction (A) inhibited growth in a dilution of 1:512,000 while the 15-percent fraction (B) and supernatant from the 15-percent precipitate (C) inhibited growth in dilutions of 1:64,000 and 1:2,560 respectively when in the presence of sodium sulfadiazine. On the basis of information concerning the β- and γ-globulin character of specific antibodies, the activity of this serum and its fractions might have been referred to these components as determined electrophoretically. However, since the primary purpose was one of comparison with the activity obtained in normal serum fractions and since the normal growth-inhibitor remains to be identified with particular serum protein components, reference of activity to the total protein seemed preferable. Therefore, the relative effectiveness of serum and its fractions was expressed as the minimum amount of total protein necessary for demonstrable action. It required a minimum of 1.8×10^{-7}, 0.8×10^{-7}, 1.1×10^{-7}, and 165×10^{-7} gm. of total protein in the whole serum and fractions A, B, and C respectively to evidence growth-inhibiting action. Fractions A, B, and C contained 512,000, 64,000 and 2,560 units respectively capable of growth-inhibiting action or 88.5, 11.0, and 0.5 percent of the antibody. Thus over 99 percent of the specific growth-inhibitor in this serum was precipitated at 15-percent sodium sulfate concentration, 89 percent of which precipitated

out at 12.5-percent sodium sulfate concentration.

c. Electrophoretic analyses. -- Electrophoretic analyses of serum 991 and its three fractions were made by Dr. R. E. Sanders from patterns obtained in the Tiselius apparatus by the Longsworth scanning method. Electrophoresis was conducted at 0.5° C. in a barbital buffer of 0.2 ionic strength and pH 8.6 at a potential gradient of 3.5 volts per cm. The time was 20,000 seconds. The protein concentration was 1.5 percent. Certain data from the ascending patterns are presented in table 9.

A correlation was noted between the γ-globulin content and the activity of fractions A and C. The lack of resolution of the globulin components in the electrophoretic patterns of fraction B and of the whole serum did not permit calculation of the γ-globulin content. The 12.5-percent fraction (A) consisted of almost pure γ-globulin as indicated by the symmetry of the single peak; fraction C contained some of each of the components present in the original serum. Since 89 percent of the growth-inhibiting activity was associated with fraction A, or γ-globulin, the specific growth-inhibiting factor would appear to migrate with the γ-globulin. Fraction A possessed 0.0414 gm. of γ-globulin and 512,000 growth-inhibiting units per ml. as compared to 0.0014 gm. of γ-globulin and 2,560 growth-inhibiting units per ml. in fraction C. A minimum of 0.8×10^{-7} and 0.6×10^{-7} gm. of γ-globulin in fractions A and C respectively produced demonstrable growth-inhibiting action. Hess and Deutsch (20) have recently reported the specific bovine bactericidin against Brucella to be in γ_1- and γ_2-globulin fractions prepared by the ethanol method.

2. Fractionation of normal serum

a. Preparation of fractions. -- By analogy with the specific serum

Table 9

Growth-Inhibiting Action and Electrophoretic Analyses of Serum and Its F

Substance	Protein[a] gm./100 ml.	Percent of original protein	Growth inhibition titer[a] 1:	Protein per unit growth inhibition gm.	Electroph	
					Relative c	
					Perce	
					A	α
Serum 991	9.28	100	5.12×10^5	1.8×10^{-7}	33.6	12.6
Fraction A	4.14	45	5.12×10^5	0.8×10^{-7}	0	0
Fraction B	0.73	8	6.40×10^4	1.1×10^{-7}	1.7	10.3
Fraction C	4.23	46	2.56×10^3	165×10^{-7}	64.1	18.8

These electrophoretic analyses were made from photographs take1 after apparatus at 0.5° C. in barbital buffer of pH 8.6 at an ionic strength o sample was 1.5 percent.

A = fraction precipitated at 12.5 percent sodium sulfate concentratio
B = fraction precipitated at 15 percent sodium sulfate concentration
C = supernatant of fraction B.
[a]Fractions diluted to original serum volume.

Table 9

tic Analyses of Serum and Its Fractions from a Brucella-Infected Cow

rowth nhi- ition iter[a] l:	Protein per unit growth inhi- bition gm.	Electrophoretic analyses, ascending pattern							
		Relative concentration				Protein			
		Percent area				gm./100 ml.			
		A	α	β	γ	A	α	β	γ
$12x10^{5}$	$1.8x10^{-7}$	33.6	12.6	←53.8→		3.12	1.17	←4.99→	
$12x10^{5}$	$0.8x10^{-7}$	0	0		100.0	0	0		4.14
$40x10^{4}$	$1.1x10^{-7}$	1.7	10.3	←88.1→		0.01	0.08	←0.64→	
$56x10^{3}$	$165x10^{-7}$	64.1	18.8	13.7	3.4	2.72	0.80	0.58	0.14

ade from photographs taken after 20,000 seconds in the Tiselius
of pH 8.6 at an ionic strength of 0.2. The protein content of the

ent sodium sulfate concentration.
it sodium sulfate concentration after removal of A.

volume.

it was anticipated that the growth-inhibitor of normal serum would be precipitated at 15-percent sodium sulfate concentration. Preliminary precipitation of a normal bovine serum at this salt concentration revealed that its activity lay in the supernatant fraction, contrary to the results obtained with the specific serum. Upon reprecipitation of the supernatant at 20-percent salt concentration, the activity was found to lie in the fraction precipitated at 20 percent. No activity was apparent in the supernatant from the 20-percent precipitate.

Subsequently, six normal bovine serums were precipitated at 15-percent and, after removal of the precipitate, reprecipitated at 20-percent concentration of sodium sulfate. Serums 1, 2, and 3 were taken from the same animal at the fourth and fifth months of pregnancy and 4 months after parturition; serums 4, 5, and 6 were from different animals. The normal serum fractions were prepared as were the specific ones with the following exceptions: the serums were processed within 24 hours of bleeding; an interval of 1 hour was allowed between the addition of sodium sulfate and removal of the precipitate; 30-percent sodium sulfate solution was used to precipitate the 15-percent fraction and to raise the salt concentration from 15 percent to 20 percent. Concentration of the supernatant from the 20-percent precipitate by evaporation at 37° C. required 10 to 24 hours depending on the degree of concentration attained.

b. Growth-inhibiting action. -- The results of the growth-inhibition determinations conducted immediately after processing the normal serum fractions are presented in table 10. In order to approximate any loss of activity in the fractions due to filtration, the whole serums had also been filtered. All of the 15-percent fractions in dilutions of 1:10 exhibited partial or complete growth-inhibition and those of serums

Table 10

Growth-Inhibiting Action of Normal Bovine Serum and I

Serum No.	Serum, whole						Fraction A[a]					Fracti	
	Protein gm./100 ml.	Dil. of serum, 1:					Original protein (%)	Dil. of A, 1:				Original protein (%)	Di
		10	20	40	80	160		10	20	40	80		1
1	7.34	1+	1+	1+	4+	4+	28.5	1+	4+	4+	4+	-	1
2	7.02	±	±	-	-	4+	26.9	2+	3+	4+	4+	10.3	
3	8.46	-	-	-	-	4+	30.4	1+	1+	4+	4+	10.0	
4	8.47	-	±	2+	4+		40.4	2+	4+	4+	5+	9.4	4
5	7.62	-	-	-	3+	4+	35.0	1+	4+	4+	4+	9.1	
6	8.49	-	-	-	4+	4+	45.2	-	-	2+	4+	6.5	4

Serums 1, 2, and 3 were obtained from the same cow at the 4th and 5th r after parturition. Serums 4, 5, and 6 were from different cows.

Each dilution in 5 ml. of culture medium also contains 10^8 Br. abortus serum, and 0.01 mg. sodium sulfadiazine. Incubation period, 72 hours. B

A = fraction precipitated at 15-percent sodium sulfate concentration.

B = fraction precipitated at 20-percent sodium sulfate concentration a

C = supernatant of fraction B.

[a]Fraction diluted to original serum volume.

- = no visible growth. + = degree of growth, turbidity.

Table 10

ion of Normal Bovine Serum and Its Fractions

Fraction A[a]		Fraction B[a]		Fraction C[a]	
inal tein %)	Dil. of A, 1: 10 20 40 80	Original protein (%)	Dil. of B, 1: 10 20 40 80	Original protein (%)	Dil. of C, 1: 10 20 40 80
8.5	1+ 4+ 4+ 4+	-	1+ - 4+ 4+	53.3	4+ 4+ 4+
6.9	2+ 3+ 4+ 4+	10.3	- - 4+ 4+	58.0	4+ 4+ 4+
0.4	1+ 1+ 4+ 4+	10.0	- - 2+ 4+	53.7	4+ 4+ 4+
6.4	2+ 4+ 4+ 5+	9.4	4+ 4+ 4+ 4+	32.5	3+ 3+ 4+
5.0	1+ 4+ 4+ 4+	9.1	- - 3+ 4+	42.5	- 3+ 4+
5.2	- - 2+ 4+	6.5	4+ 4+ 4+ 4+	42.2	5+ 5+ 5+

he same cow at the 4th and 5th month of pregnancy and 4 months
e from different cows.
m also contains 10^8 Br. abortus, 0.2 ml. of fresh, normal rabbit
Incubation period, 72 hours. Bacterial controls, 4+ growth.
sodium sulfate concentration.
sodium sulfate concentration after removal of A.

ume.
rowth, turbidity.

3 and 6 were slightly active in dilutions of 1:20 and 1:40 respectively. Of the 20-percent fractions, four completely inhibited growth in a 1:20 dilution but two showed no activity. One 20-percent supernatant, No. 5, inhibited growth at a 1:10 dilution and evidenced a very slight, or no, action at 1:20 as did supernatant 4 in dilutions of 1:10 and 1:20. The other supernatants were inactive. Tests on the 15- and 20-percent fractions at 3 or 4 times the original concentration confirmed the degree of activity in table 10 and failed to reveal any in the 20-percent fraction of serum 4 although that of serum 6 showed a slight activity.

The relative effectiveness of normal serum and its fractions, expressed as the minimum of total protein necessary for demonstrable action, was 0.1-0.2 gm. in the whole serum, 0.2-0.4 gm. in the 15-percent fraction, 0.03-0.04 gm. in the 20-percent fraction, and 0.3 gm. in the 20-percent supernatant. These are approximations: for example, only partial inhibition of growth was shown by the 1:10 dilutions of some 15-percent fractions but a titer of 1:10 was assumed in the above calculations. No attempt was made to estimate the percent of total activity precipitated with each fraction of normal serum. The small differences in activities did not warrant an evaluation of purification on this basis. However, since the protein necessary for demonstrable activity by the 20-percent fraction of four out of six serums was one-third to one-fifth that of the whole serum and other fractions, a certain degree of purification had been effected in these cases.

If one considers both the percent of original protein and growth-inhibiting activity of fractions from serums 1, 2, and 3 (from the same animal), the salting-out procedure appears to have given fairly reproducible fractions. The protein content of the 15-percent fraction from

different animals varied from 27 to 46 percent of the original protein. The difference is not surprising in view of reports of individual variations in the normal bovine albumin component alone of values from 35 to 53 percent (27). However, it may be noted that the two serums, Nos. 4 and 6, which showed no activity in the 20-percent fraction were those from which 45 to 46 percent of the original protein precipitated with the 15-percent fraction. The significance of this is not clear. The protein content of the 20-percent fractions varied from 6 to 10 percent of the original protein.

The normal brucella growth-inhibiting factor was irregularly distributed over the serum fractions obtained by precipitation at 15-percent and reprecipitation at 20-percent concentrations of sodium sulfate. The distribution varied with serum from different animals. All the demonstrable growth-inhibiting factor of serums from two animals precipitated at 15-percent sodium sulfate concentration and was associated with 45 to 46 percent of the original protein. The major part of the factor in serum from three out of five animals precipitated with the 20-percent fraction which contained 10 percent, or less, of the original protein.

c. Electrophoretic analyses. -- Identification of the major part of the activity with a fraction low in protein led to the electrophoretic examination of serum 3 and its fractions. Analyses were made by Dr. R. E. Sanders from patterns obtained in the Tiselius apparatus. Electrophoresis was conducted under the same conditions as for the specific serum. Certain data from the ascending pattern are presented in table 11. Resolution of the boundaries on the ascending side permitted calculation of an additional component in one fraction; in other respects the ascending and descending patterns were in close agreement.

Table 11

Growth-Inhibiting Action and Electrophoretic Analyses of a Normal Bov

Substance	Protein[a] gm./100 ml.	Percent of original protein	Growth-inhibition titer[b] 1:	Protein per unit growth-inhibition gm.	Electrophoretic			
					Relative concentrat			
					Percent area			
					A	α	β_1	β_2
Serum 3	8.46	100	80	0.11	51.2	12.0	8.4	←28
Fraction A	2.57	30	<20	>0.12	4.8	9.9	←—	85.4
Fraction B	0.85	10	20	0.04	17.8	42.9	8.4	18.1
Fraction C	4.54	54	None		78.1	13.5	8.5	

These electrophoretic analyses were made from photographs take1 after 20 apparatus at 0.5° C. in barbital buffer of pH 8.6 at an ionic strength of 0 samples was 1.5 percent.

A = fraction precipitated at 15-percent sodium sulfate concentration.
B = fraction precipitated at 20-percent sodium sulfate concentration aft
C = supernatant of fraction B.
[a]Fractions diluted to original serum volume.
[b]See table 10.

Table 11

horetic Analyses of a Normal Bovine Serum and Its Fractions

Protein per unit growth-inhibition gm.	Electrophoretic analyses, ascending pattern									
	Relative concentration					Protein				
	Percent area					gm./100 ml.				
	A	α	β_1	β_2	γ	A	α	β_1	β_2	γ
0.11	51.2	12.0	8.4	←28.4→		4.33	1.02	0.71	←2.40→	
>0.12	4.8	9.9	←——85.4——→			0.12	0.25	←——2.19——→		
0.04	17.8	42.9	8.4	18.1	12.8	0.15	0.36	0.07	0.15	0.11
	78.1	13.5	8.5			3.55	0.61	0.39		

from photographs take1 after 20,000 seconds in the Tiselius
pH 8.6 at an ionic strength of 0.2. The protein content of the

sodium sulfate concentration.
sodium sulfate concentration after removal of A.

ume.

Examination of the data reveals that the inactive 20-percent supernatant fraction (C) contained a higher concentration of albumin and α- and β_1-globulin than the active 20-percent fraction (B). Fraction C contained 3.55, 0.61, and 0.39 gm. per 100 ml. of albumin, α-, and β_1-globulin respectively as compared to 0.15, 0.36, and 0.07 gm. per 100 ml. of these components in fraction B. On the other hand, the inactive fraction C possessed neither β_2- nor γ- globulins both of which were present in the active fraction B. Assuming no qualitative differences in the common components, one is lead to infer that the growth-inhibitor may migrate with the β_2- or γ-globulins which were absent in the inactive and present in the active fractions. As the broad γ-globulin peak of the whole serum and that of the 15-percent fraction did not permit estimation of the individual β_2- and γ-areas, no calculation of the percent precipitated in the 20-percent fraction was possible. However, the activity does not appear to be a function of total γ-globulin as the 20-percent fraction was slightly more active than the 15-percent fraction which contained more than 90 percent of the total γ-globulin.

3. Comparison of specific and normal serum fractions

The failure of the normal growth-inhibiting factor of different serums to precipitate uniformly prevented a comparison of the salt solubility of the specific and normal factor as originally intended. It would appear, from the one specific serum examined, that 99 percent of the growth-inhibitor was precipitated at 15-percent sodium sulfate concentration, whereas the major part of the activity in three out of five normal serums was not precipitated at this salt concentration. The low growth-inhibition titer encountered in normal serums does not permit calculation of the amount present when the factor appears in more than

one fraction. Additional investigation might establish a solubility differential of the factors. The differences observed in serums from only five normal animals indicate that this would necessitate a considerable number of determinations for significant data and entail a study in itself. It has been assumed that the antibody of specific serums would precipitate in a uniform manner, a conclusion which possibly is not justified although the antibacterial antibodies have generally been found to be insoluble at 15-percent sodium sulfate concentration.

The data in tables 9 and 11 show that the concentration of serum components in the normal and specific serums was vastly different; the former contained 4.3 gm. of albumin and 4.1 gm. of globulin per 100 ml. and the latter 3.1 gm. of albumin and 6.2 gm. of globulin per 100 ml. Different amounts of protein might be expected to precipitate from these two serums at identical salt concentrations. Nevertheless, the wide range in concentration of normal bovine serum components (27) does not permit a comparison of fractions from relatively few serums on this basis.

The electrophoretic and growth-inhibiting data in tables 9 and 11 indicate that the specific antibody is a function of total γ-globulin while the normal growth-inhibiting factor is not. The 12.5-percent fraction of specific serum 991 with which 89 percent of the activity was associated comprised more than 80 percent of the total γ-globulin. The major part of the activity of normal serum 3 was present in the 20-percent fraction which contained 5 percent of the total γ-globulin.

Since development of the fractionation procedure resulted in fractions of specific and normal serum precipitated at different limits,

the electrophoretic patterns were not strictly comparable. It was decided to compare identical fractions. A comparison of fractions precipitated at, or below, 15-percent salt concentration seemed of limited value as the β- and γ-globulin boundaries of these specific serum fractions had not been resolved. Therefore, a fraction of specific serum 991 was precipitated at 20-percent sodium sulfate concentration after removal of the material precipitated at 15-percent and compared with the corresponding fraction of normal serum 3. Electrophoresis was conducted under the same conditions as previously stated.

The data presented in table 12 show that 0.85 gm. and 0.38 gm. of protein or 10 percent and 4 percent of the original protein precipitated from serums 3 and 991 respectively. As may be noted in table 10, from 6 to 10 percent of the original protein precipitated from five fresh, normal serums under these same conditions. One year had elapsed between the preparation of the original fractions from serum 991 and this particular fraction; during storage of the whole serum a small amount of material precipitated and the protein fell from 9.28 percent to 8.95 percent. In view of the variation in protein content of the 20-percent fraction from normal serums and in view of the loss of undefined protein during storage of serum 991, the significance of the low protein content of the specific serum is uncertain. No correlation existed between the absolute concentration of serum components and the growth-inhibiting activity. The normal serum fraction was approximately twice as concentrated in each component as the specific serum fraction although it contained only 20 growth-inhibiting units per ml. as compared with 2,560 units per ml. in the specific serum fraction.

The albumin-globulin ratios of the whole serums were 0.5 and 1.0.

Table 12

Growth-Inhibiting Action and Electrophoretic Analyses of
from a Normal Cow and from a Brucella-Infecte

Serum No.	Serum source	Protein[a] gm./100 ml.	Percent of original protein	Growth-inhibition titer 1:	Electrophore		
					Relative conce		
					Percent a		
					A	α	
3	Normal cow	0.85	10	20	17.8	42.9	2(
991	Infected cow	0.38	4	2560	17.0	39.4	3(

[1]The fraction of serum insoluble in 20 percent sodium sulfate but solu

[a]Fraction diluted to original serum volume.

Table 12

nd Electrophoretic Analyses of a Serum Fraction[1]
Cow and from a Brucella-Infected Cow

ıt ıal ın	Growth-inhibition titer 1:	Electrophoretic analyses, ascending pattern							
		Relative concentration				Protein			
		Percent area				gm./100 ml.			
		A	α	β	γ	A	α	β	γ
	20	17.8	42.9	26.5	12.8	0.15	0.36	0.22	0.11
	2560	17.0	39.4	30.9	12.7	0.06	0.15	0.12	0.05

percent sodium sulfate but soluble in 15 percent sodium sulfate.

lume.

Nevertheless, the relative concentrations of components in the 20-percent fraction from the normal and specific serum were in very good agreement. That from normal serum 3 contained 17.8 percent albumin and 42.9, 26.5, and 12.8 percent of α-, β-, and γ-globulin respectively. That from specific serum 991 contained 17.0 percent albumin and 39.4, 30.9, and 12.7 percent of α-, β-, and γ-globulin respectively. The relative concentrations appear to be independent of the albumin-globulin ratios of whole serum.

As already mentioned, the most distinctive difference observed between the specific and normal serums studied was that of solubility. This was demonstrated again in the comparison of the 20-percent fractions from a specific and from a normal serum. While the 20-percent fraction from specific serum 991 contained 2,560 units of growth-inhibitor, it represented only 0.5 percent of the total activity in the serum fractions. On the other hand, the 20 units in the 20-percent fraction from normal serum 3 represented over 50 percent of the total growth-inhibiting activity demonstrable. In both instances the remainder of the activity was present in the 15-percent fraction.

E. The Effect of Certain Physical Forces on the Brucella Growth-Inhibiting Action of Bovine Serum, Plasma, and Colostrum

1. The effect of heat

a. Normal serum and serum fractions. -- Although the bactericidal phenomenon has been known since 1888, the dual role of antibody and complement in the action of normal serum was not proved for many years (12), (28). As a result, few data pertaining to the effect of heat on the normal bactericidal antibody independent of complement are available. Mackie and Finkelstein (12) investigated the thermostability of normal bactericidins against organisms of the typhoid-paratyphoid group and found them to correspond to the natural agglutinins in heat stability since they were generally stable at 55° C. and inactivated at 60° to 65° C. The activity of natural complement-fixing antibodies, other than the bactericidins, which are capable of reacting with bacterial antigens usually is annulled at 55° C. (23).

In order to determine the effect of heat on the growth-inhibiting action of normal bovine serum, 18 serums from different animals were examined. The group was comprised of 13 cows, 3 newborn calves, 1 seven-week-old calf and 1 one-year-old calf; two of the cows were bled more than once. The serums were heated at 56° C. for 30 minutes on the day of testing. The growth-inhibiting activity of the heated serum was compared with that of the unheated serum in the presence of sodium sulfadiazine. The results demonstrate that the growth-inhibitor of normal bovine serum resembles other natural complement-fixing antibodies in being labile at 56° C.

As may be observed from the data in table 13, the activity of 10

Table 13

Effect of Heat on the Growth-Inhibiting Action of Normal Bovine Serum

Serum and animal No.	Degree of growth, turbidity[a]											
	Unheated serum						Heated serum[b]					
	Dilution of serum, 1:						Dilution of serum, 1:					
	10	20	40	80	160	320	10	20	40	80	160	320
1, cow	±	-	-	-	±	4+	3+	±	±	4+	4+	
2, cow	-	C	-	-	-	3+	4+	1+	1+	3+	4+	4+
3, cow	-	-	-	-	3+	3+	4+	4+	4+	4+	4+	4+
4, cow	-	-	-	3+	4+	4+	4+	3+	2+	2+	C	4+
5, cow	2+	1+	±	-	-	3+	4+	4+	4+	4+	4+	4+
6, cow	-	-	-	-	-	4+	3+	-	-	2+	C	4+
7, cow	-	-	-	-	4+	4+	2+	-	-	4+	4+	4+
8, cow	1+	1+	-	4+	4+	4+	4+	C	4+	4+	4+	4+
9, cow	-	-	-	3+	4+	4+	4+	4+	4+	4+	4+	4+
10, cow	-	-	±	±	5+	5+	3+	2+	4+	4+	4+	4+
11, cow	-	±	±	4+	4+		4+	4+	4+	4+	4+	
12, cow	-	-	-	-	4+		5+	5+	5+	5+	5+	
13, cow	-	-	-	-	2+		5+	3+	4+	5+	5+	
14, calf[c]	-	-	3+				4+	4+	4+			
15, calf[c]	-	-	3+	4+			4+	4+	4+	4+		
16, calf[c]	4+	2+	3+	4+	5+		4+	4+	4+	4+	4+	
17, calf[d]	-	4+	4+	4+	4+		6+	6+	6+	6+		
18, calf[e]	3+	C	-	-	3+		5+	4+	2+	3+	4+	

[a] 10^5 Br. abortus, 0.2 ml. fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours. Bacterial controls, 4+ growth.

[b] Serum heated at 56° C. for 30 minutes. [c] Newborn calf, before colostrum. [d] 7-week-old calf. [e] 1-year-old calf.

C = contamination. - = no visible growth. + = degree of growth.

serums was completely destroyed and that of eight serums was reduced by heating at 56° C. The eight serums with residual activity showed a zone reaction which varied from one with complete inhibition of growth in certain dilutions to one represented only by different degrees of turbidity. There appeared to be no relationship between the initial presence of a zone and the result of heating. Of the eight serums exhibiting a zone reaction after heating, only serum 18 originally showed a definite zone and serum 1 a doubtful zone. Of the 10 serums whose activity was completely destroyed by heat, serums 5 and 8 showed a definite zone and serum 16 a slight zone before heating.

The serums obtained from two of these cows on other occasions showed the effect of heat to be reproducible; heated serum from animal 1 again displayed a similar reduction in activity and a prozone growth; serum from animal 3 was inactivated by heating at 56° C. for 30 minutes.

To determine the thermolability of the normal growth-inhibitor in serum fractions, three concentrated fractions were heated at 56° C. for 30 minutes, and the activities were compared with those of the unheated fractions. The results in table 14 show that heating reduced the growth-inhibiting action of the serum fractions. After heating, fraction 1 exhibited no activity, and fraction 2 showed a very slight inhibition only in the 1:80 dilution. Heating of fraction 3 reduced the titer from 1:80 to 1:40 and produced a definite prozone reaction. Although purification is known to affect the stability of many substances, partial purification apparently did not alter the resistance of the normal growth-inhibitor to heat. The reduction in activity of the serum fractions and the degree of prozone growth were of the same order as that which resulted from the heating of whole serum.

Table 14

Effect of Heat on the Growth-Inhibiting Action of Normal Bovine Serum Fractions

Fraction No.	Serum fraction	Degree of growth, turbidity[a]					
		Dil. of fraction, 1:					Bacterial control
		10	20	40	80	160	
1	A[c], unheated	2+	1+	2+	4+	4+	4+
	A[c], heated[d]	4+	4+	4+	4+	4+	
2	B[b], unheated	3+	3+	1+	1+	4+	4+
	B[b], heated[d]	4+	4+	4+	3+	4+	
3	B[b], unheated	-	-	-	-	4+	4+
	B[b], heated[d]	4+	1+	-	2+	4+	

A = fraction precipitated at 15-percent sodium sulfate concentration.

B = fraction precipitated at 20-percent sodium sulfate concentration after removal of A.

[a] 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Concentrated to one-quarter the original serum volume.

[c] Concentrated to one-third the original serum volume.

[d] Serum fraction heated at 56° C. for 30 minutes.

- = no visible growth. + = degree of growth.

b. Specific antiserum. -- The thermostability of the growth-inhibitor in serum from one brucella-infected cow was examined. Serum 991 (agglutination titer of 1:20,480) was heated at 56° C. for 30 minutes in the undiluted state, diluted 1:100 in 0.85-percent sodium chloride solution, and diluted 1:100 in a normal serum free from growth-inhibiting activity. The growth-inhibiting activities of the heated and unheated serum were compared in the presence of sodium sulfadiazine. The results in table 15 show that the growth-inhibition titer (1:256,000) was not affected by heating nor was the extent of the prozone reaction appreciably altered. It had been thought that the effect of heat on the activity in diluted serum might be more pronounced than on that in undiluted serum, but no difference was apparent. The relative thermostability of specific antibodies as compared with natural ones which exhibit the same reaction is well established (29). This distinction may be seen in a comparison of the effect of heat on the growth-inhibiting activity of normal serum and specific antiserum. The specific growth-inhibitor, unlike the natural factor, was heat stable at 56° C. for 30 minutes when tested in the presence of sodium sulfadiazine.

c. Plasma fractions. -- The effect of heat on the growth-inhibiting action of certain Armour and Company bovine plasma fractions was investigated. It must be stressed that the status of the animals which contributed to the pooled, dried plasma was unknown. However, an agglutination titer of 1:80 in a 2-percent solution of fraction II from bovine plasma suggested the presence of specific antibody, and it seemed probable that the antibody might prove to be stable at 56° C. The results substantiated this. One sample was heated 30 minutes and another sample 150 minutes. It is interesting to note in table 16 that even heating for

Table 15

Effect of Heat on the Growth-Inhibiting Action of Serum from a Brucella-Infected Cow

Serum	Degree of growth, turbidity[a]										
	Dilution of serum, 1:										
	1.0×10^3	2.0×10^3	4.0×10^3	8.0×10^3	1.6×10^4	3.2×10^4	6.4×10^4	1.28×10^5	2.56×10^5	5.12×10^5	Bacterial control
Unheated	4+	4+	4+	4+	3+	1+	-	-	-	1+	4+
Heated[b], undiluted	4+	4+	4+	4+	3+	2+	1+	1+	-	3+	4+
Heated[b], diluted 1:100 in saline	4+	4+	4+	4+	3+	2+	1+	-	-	2+	4+
Heated[b], dil. 1:100 in negative serum	3+	4+	4+	4+	2+	1+	±	-	-	1+	4+

[a] 10^5 Br. abortus, 0.2 ml. fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Serum heated at 56° C. for 30 minutes.

- = no visible growth. + = degree of growth.

Table 16

Effect of Heat on the Growth-Inhibiting Action of Armour and Company's Fractions from Bovine Plasma

Fraction	Concentration (percent)	Degree of growth, turbidity[a]										
		Dilution of sample, 1:										Bacterial control
		1.0×10	2.0×10	4.0×10	8.0×10	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	
II, unheated	0.2	4+	2+	-	-	-	-	C	-	2+	4+	4+
II, heated[b] 30 min.	0.2	4+	3+	-	-	-	-	-	-	3+	4+	4+
II, heated[b] 150 min.	0.2	4+	3+	-	-	-	-	-	-	2+	4+	4+
IV, unheated	1.0	±	-	-	-	1+	4+	4+				4+
IV, heated[b] 30 min.	1.0	-	-	-	-	3+	4+	4+				4+

[a] 10^5 Br. abortus, 0.2 ml. fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Fraction heated at 56° C.

C = contamination. - = no visible growth. + = degree of growth.

150 minutes failed to alter the growth-inhibition titer or the prozone reaction of the plasma fraction.

Data on the effect of heat on the activity of fraction IV are included in table 16. It was not possible to obtain a clear 1-percent solution of fraction IV in 0.85-percent sodium chloride solution. The cloudy suspension, therefore, was filtered through a Hormann D8 pad in a Seitz filter which resulted in a clear filtrate of undetermined concentration. This sample failed to show agglutinins in a dilution of 1:20 or to inhibit growth in dilutions greater than 1:80. Although the growth-inhibition titer of fraction IV was the same as that of the average normal serum, heating at 56° C. for 30 minutes did not significantly reduce its activity. The growth-inhibiting factor of bovine plasma fractions II and IV thus resembled that of specific antiserum in its thermostability.

d. Normal colostrum. -- Comparative data on the growth-inhibiting activity of heated and unheated colostrum in the presence of complement and sodium sulfadiazine are set forth in table 8. The results show that heating produced little effect on the activity of colostrums 1, 3, 4, and 5, slightly reduced the activity of colostrums 2, 6, and 8, and markedly lowered that of colostrum 7. Colostrums 1, 2, 4, and 7 evidenced a slight prozone reaction after heating. Heating produced a similar varied reduction in the potency of the colostral principle capable of growth-inhibition in the presence of sodium sulfadiazine without the presence of complement (table 8).

A consideration of the pronounced difference in activity between heated and unheated normal serums, in the presence of sodium sulfadiazine and complement, and the comparatively slight effect upon exposing

colostrum to the same conditions indicates a difference in thermostability between the normal brucella growth-inhibitor of serum and that of colostrum.

2. The effect of storage

a. Normal serum -- In view of the marked thermolability of the normal growth-inhibitor of serum, the effect of storage on its activity was investigated. Irwin et al. (13) found the bactericidal activity of bovine serums stored at 3° C., as determined without sulfonamides and without added complement, to decrease until at 28 days little or none was apparent. The activity was restored by the addition of bovine serum complement. Likewise, Huddleson et al. (3) reported a loss in bactericidal action in 30 days, presumably due to deterioration of the complement. A survey of the literature revealed no investigation of the effect of extended storage on the natural antibody independent of complement.

Seven normal bovine serums were stored at 4° C., and the growth-inhibiting action, in the presence of sodium sulfadiazine and added complement, was estimated at various intervals. Serums 4 and 5 were from the same cow at the fourth and fifth months of pregnancy; the other serums were from different animals. The data in table 17 illustrate the effect of storage on the natural brucella growth-inhibiting factor. Serums 1 and 2 failed to inhibit growth significantly in dilutions of 1:10 or greater after storage for 9 and 8 weeks respectively. At 10 weeks serum 3 was active in a dilution of 1:20 as compared with an original activity in a dilution of 1:40. When tested at 20 weeks, no growth-inhibition was evidenced by serum 3 although it exhibited partial inhibition in dilutions of 1:10 and 1:20 at 22 weeks. Serum 4

Table 17

Effect of Storage on the Growth-Inhibiting Action of Normal Bovine Serum

Serum No.	Storage at 4°C. (weeks)	Degree of growth, turbidity[a]					
		Dilution of serum, 1:					Bacterial control
		10	20	40	80	160	
1	1	-	-	-	4+	4+	4+
	9	4+	4+	4+	4+	5+	4+
	23	4+	3+	4+	4+	4+	4+
2	0	-	1+	2+	4+		4+
	8	3+	3+	4+	C		
	12	4+	4+	4+	4+		4+
	21	4+	4+	4+	4+		4+
3	0	-	-	-	4+	4+	4+
	10	-	-	3+	4+		4+
	20	4+	4+	4+	4+		4+
	22	2+	1+	4+	4+		4+
4	1	-	-	-	4+		4+
	9	-	-	-	3+		4+
	13	±	-	4+	5+		4+
	22	4+	4+	4+	4+		4+
5	0	1+	1+	-	3+	4+	4+
	4	3+	2+	4+	4+	4+	
	8	1+	-	3+	4+		4+
	18	3+	2+	4+	4+		4+
	20	4+	3+	4+	4+		4+
6	0	-	-	-	-	4+	4+
	6	±	±	-	4+	4+	4+
	20	-	-	4+	4+	4+	4+
7	1	-	-	-	4+	4+	4+
	16	±	-	3+	5+		4+
	17	2+	-	4+	4+	4+	
	28	3+	±	4+	4+		

[a] 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

- = no visible growth. + = degree of growth.

C = contamination.

exhibited activity in a dilution of 1:20 at 13 weeks as compared with original activity in a 1:40 dilution; this serum failed to inhibit growth in a dilution of 1:10 or greater after 22 weeks. Serum 5 showed reduced activity during storage with evidence of the original prozone reaction throughout the time; no significant activity remained at 20 weeks. Serums 6 and 7 were active in a dilution of 1:20 at 20 and 28 weeks respectively although the latter showed prozone growth in a dilution of 1:10 and the original activity of both serums was reduced.

It must be stressed that the growth-inhibiting activities determined at different times are not strictly comparable. Analyses were necessarily conducted with different rabbit serums as a source of complement and different bacterial suspensions. The discrepancy in the growth-inhibiting activity of serum 3 at 20 and 22 weeks is obvious. The culture used for the determination at 20 weeks was suspected of dissociation. A second determination at 22 weeks was made with a culture derived from a selected colony. Serum 5 also presented a picture of less activity after 4 weeks than after 8 weeks of storage. These irregularities emphasize the fact that interpretation of the effect of storage on growth-inhibiting activity should be based on general trends and not on absolute values.

The foregoing results show that some normal bovine serums lost their growth-inhibiting activity, as determined in the presence of sodium sulfadiazine and added complement, within 2 months and others within 5 months of storage. Some serums remained active after 5 and 7 months of storage although the growth-inhibition titer was reduced.

b. Normal serum fractions. -- The effect of storage for 5 months at 4° C. on the growth-inhibiting activity of normal serums and

their fractions was determined. The activities of one representative serum and its fractions are recorded in table 18. Data were obtained on samples of serum fractions diluted to original serum volume and also on concentrated samples so that any residual activity might be apparent. The growth-inhibition titer of the whole serum declined from 1:80 to 1:20 in 8 weeks, and that of fraction B declined from 1:20 to 1:10; the activity of fraction A remained essentially unchanged. However, after 5 months of storage the fractions failed to exhibit activity although a very slight zone action was evident in the whole serum.

Storage at 4° C. affected three other serums and their fractions in a similar manner. At 3 months a diminished activity still remained in the fractions but at 5 months this had disappeared. Two of the three whole serums were still slightly active at 5 months.

c. Normal colostrum. -- Table 19 illustrates the effect of storage at different temperatures on the growth-inhibiting activity of a representative colostral whey in the presence of sodium sulfadiazine. Five wheys were stored at room temperature, 4° C., and -10° C. and examined for growth-inhibiting activity after 6, 11, and 22 weeks. After 6 weeks at room temperature some reduction in the growth-inhibiting activity of three of the wheys was apparent. A definite loss in activity was evident in four of the colostrums held at room temperature for 11 weeks; the colostrum which had the highest original titer retained its activity. Unfortunately, the appearance of mold in the samples held at room temperature prevented analyses at 22 weeks. Although slight deviations from the original activity occurred in samples stored for 22 weeks at 4° C. or -10° C., they did not exceed the variables of the test. It may be concluded that the brucella growth-inhibiting activity of colostrum,

Table 18

Effect of Storage on the Growth-Inhibiting Action of a Normal Bovine Serum and Its Fractions

Storage[b] (mo.)	Degree of growth, turbidity[a]				
	Serum, whole	Fraction A		Fraction B	
	Conc., orig.	Conc., orig.	Conc.,3 times	Conc., orig.	Conc.,4 times
	Dil. of serum, 1:	Dil. of A, 1:	Dil. of A, 1:	Dil. of B, 1:	Dil. of B, 1:
	10 20 40 80 160	10 20 40 80	10 20 40 80	10 20 40 80	10 20 40 80 160
0	± ± - - 4+	2+ 3+ 4+ 4+	2+ 1+ 4+ 4+	- - 4+ 4+	- - - - 4+
2	1+ - 3+ 4+	2+ 4+ 4+ 4+	2+ - 2+ 4+	- 3+ 4+ 4+	4+ - - 3+
5	4+ 3+ 4+ 4+		4+ 4+ 4+ 4+		4+ 4+ 4+ 4+

A = fraction precipitated at 15-percent sodium sulfate concentration.
B = fraction precipitated at 20-percent sodium sulfate concentration after removal of A.

[a] 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours. Bacterial controls, 4+ growth.

[b] Stored at 4° C.

- = no visible growth. + = degree of growth.

Table 19

Effect of Storage at Different Temperatures on the Growth-Inhibiting Action of Colostral Whey in the Presence of Complement

Storage time (weeks)	Degree of growth, turbidity[a]														
	Temp., room					Temp., 4° C.					Temp., -10° C.				
	Dil. of whey, 1:					Dil. of whey, 1:					Dil. of whey, 1:				
	10	20	40	80	160	10	20	40	80	160	10	20	40	80	160
0						-	-	2+	2+	3+					
6	1+	3+	3+	4+	4+	-	-	3+	4+	4+	-	-	3+	4+	4+
11	4+	4+	4+	4+		-	-	4+	4+		-	-	4+	4+	
22						-	1+	4+	5+		1+	1+	3+	4+	

[a] 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours. Bacterial controls, 4+ growth.
- = no visible growth. + = degree of growth.

in the presence of sodium sulfadiazine and complement, was lowered by exposure to room temperature for several weeks but was not significantly reduced by storage at 4° C. or -10° C. for 5 months. It proved to be less affected by storage than the factor in serum or serum fractions, which parallels the effect of heat on their respective activities.

Since growth-inhibition occurred in colostrum without the presence of complement, the ability to inhibit Brucella in the presence of sodium sulfadiazine alone (without complement) was examined after storage at 4° C. for 5 months. Inspection of table 20 shows that, with the exception of whey 2, the complement-independent growth-inhibiting factor retained a large proportion of its activity through 5 months of storage.

3. The effect of filtration

a. Normal serum. -- Repeated experiments by Huddleson et al. (3) revealed that filtration through a Hormann pad brought about an increase in the bactericidal action of normal bovine plasma. In this study filtration was found to reduce the growth-inhibiting action of normal serum in the presence of sodium sulfadiazine.

Samples of serum, or plasma, from 11 different animals were filtered through Hormann D8 pads in Seitz filters and their growth-inhibiting activities were compared with those of the unfiltered specimens. Samples 1 through 9 were filtered once. Samples 10 and 11 were subjected to seven successive filtrations through fresh filter pads each time. Three samples were examined on the day of bleeding of the animal and eight after a short storage of the filtered and unfiltered samples under identical conditions.

The results are recorded in table 21. It is apparent that in each case the filtered serums and plasma possessed less activity than the

Table 20

Effect of Storage on the Colostral Growth-Inhibitor Not Requiring Complement

Colostrum No.	Degree of growth, turbidity[a]		
	Fresh	Stored[b], 5 mo.	
	Dil. of whey, 1:	Dil. of whey, 1:	Bacterial control
	10 20 40 80	10 20 40 80	
1	- 2+ 4+ 5+	- 4+	4+
2	± 2+ 4+ 4+	5+ 5+	4+
3	- - 2+ 4+	- 2+	4+
4	- 2+ 4+	- 4+	4+
5	1+ 4+ 4+ 4+	- 5+	4+

[a] 10^5 Br. abortus and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours.

[b] Stored at 4° C.

- = no visible growth. + = degree of growth.

Table 21

Effect of Filtration on the Growth-Inhibiting Action of Normal Bovine Serum and Plasma

Sample No.	Sample	Filtration[b]	Degree of growth, turbidity[a]					
			Dilution of sample, 1:					
			10	20	40	80	160	320
1	Plasma	None	-	-	-	-	4+	
		1	-	-	-	1+	4+	
2	Serum	None	-	-	-	3+	4+	
		1	-	1+	2+	4+		
3	Serum	None	-	-	-	-	-	4+
		1	-	-	-	-	4+	4+
4	Serum	None	-	-	-	-	4+	4+
		1	-	-	-	4+	4+	4+
5	Serum	None	-	-	-	-	2+	
		1	-	-	-	-	4+	
6	Serum	None	-	-	-	1+	5+	
		1	-	-	-	4+	5+	
7	Serum	None	2+	1+	±	±	4+	
		1	2+	1+	1+	4+	5+	
8	Serum	None	-	-	4+			
		1	-	3+	4+			
9	Serum	None	-	-	2+	4+	5+	
		1	2+	3+	4+	5+	5+	
10	Serum	None	-	-	-	-	4+	
		1	-	-	2+	4+	4+	
		7	1+	±	3+	4+	4+	
11	Serum	None	-	-	-	-	2+	
		1	-	-	1+	2+	5+	
		7	3+	2+	2+	5+	5+	

[a] 10^6 Br. abortus, 0.2 ml. fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine added to each dilution and to controls (5 ml.). Incubation period, 72 hours. Bacterial controls, 4+ growth.

[b] Filtration through a Hormann D8 pad in a Seitz filter.

- = no visible growth. + = degree of growth.

unfiltered ones. Although the growth-inhibiting activities of plasma 1 and serums 5 and 6 were only slightly reduced by one filtration, the other samples required 2 to 4 times as great a concentration of once-filtered serum as of unfiltered serum to inhibit the growth of Br. abortus. While seven filtrations of serums 10 and 11 removed more growth-inhibitor than did one filtration, the activity was not reduced in proportion to the number of filtrations. It is possible that this indicates the presence of one antibody factor which was removed by filtration and another which was relatively unaffected by filtration.

b. Normal colostrum. -- Investigation of the effect of filtration on the growth-inhibiting action of colostrum was limited to one sample and, as such, can only be presented as indicative. Following seven consecutive filtrations of the whey through a Hormann D8 pad in a Seitz filter, the activity of the filtrate was determined in the presence of sodium sulfadiazine alone and with complement.

The growth-inhibiting substance capable of action without complement showed, in the presence of sodium sulfadiazine, a titer of 1:20 both before and after filtration. Repeated filtration did not impair the activity of the growth-inhibiting substance capable of action without complement.

In the presence of complement and sodium sulfadiazine, the unfiltered colostrum displayed a titer of 1:40 as compared with only partial inhibition in each dilution through 1:40 by the filtered colostral whey. The significance of these results is not clear since complete inhibition was evidenced in dilutions of 1:10 and 1:20 without the rabbit serum agent. It is conceivable that the added rabbit serum provided nutritional enrichment thereby allowing growth in dilutions showing inhibition

with sodium sulfadiazine alone. Additional data would be desirable.

F. The Effect of Normal Bovine Serum and Colostrum on the Prozone Growth-Inhibiting Action of Serum from a Brucella-Infected Cow

The recognition of the prozone antibody phenomenon in the blood serum of brucella-infected animals and the ability of fresh, normal rabbit serum in the presence of sodium sulfadiazine to inhibit it *in vitro* and *in vivo* in guinea pigs (1) disclosed a potential therapeutic agent against brucellosis. It was shown that the maintainance of a bactericidal state in brucella-infected guinea pigs by the above agents for a sufficient length of time brought about a rapid and complete termination of the infection.

The normal serum factor responsible for prozone-inhibition in the presence of sodium sulfadiazine has not been identified. It was thought of interest to examine bovine serum and colostrum for *in vitro* prozone-inhibiting activity and, if demonstrable, to determine its correlation with the growth-inhibiting activity.

1. Prozone-inhibiting action

Repeated experiments had invariably shown serum 991 from a brucella-infected cow to be capable of growth-inhibition in high dilutions but not in relatively low ones (under 1:4,000) when in the presence of sodium sulfadiazine. In order to facilitate determinations, a 1:100 dilution of this serum was chosen as representative, and the prozone-inhibiting activity of normal serum, fractions from serum, and colostrum was estimated with a 1-tube test. The sample to be tested was added in the stated amounts to a 1:100 dilution of specific serum 991 in 5 ml. of liquid culture medium. The reagents of the sulfadiazine-antibody-complement test were added, and the degree of growth estimated at 72

hours. Growth in a 1:100 dilution of the specific serum had been shown to be 4+ or 5+ on innumerable occasions; therefore, less than 4+ growth in the presence of added substances indicated prozone-inhibition.

a. Normal serum. -- A preliminary examination demonstrated that normal bovine serum in a dilution of 1:10 was able to inhibit prozone growth when in the presence of sodium sulfadiazine. Additional information was sought as to the minimum concentration of normal serum required to exhibit this action. The 1-tube prozone-inhibition test was performed with the addition of 0.5 ml., 0.25 ml., and 0.1 ml. amounts of normal serum which resulted in dilutions of approximately 1:10, 1:20, and 1:50 respectively of the serum. The data set forth in table 22 show that five of the six fresh serums prevented the prozone growth when present in dilutions of 1:10. Serum 2 proved capable of inhibiting prozone growth in dilutions through 1:50. Serum 6 completely inhibited prozone growth in a dilution of 1:50 but not in dilutions of 1:10 or 1:20. Serums 3, 4, and 5 did not completely inhibit prozone growth when diluted more than 1:10.

While the results showed fresh normal bovine serum to possess prozone-inhibiting action in dilutions of 1:10 or greater, this activity and the ability to inhibit growth per se could not be examined for correlation. Unfortunately, an appreciation of the effect of filtration and storage on the growth-inhibiting action was lacking at the time this experiment was started, and each serum was not examined on the day of bleeding nor was a distinction made between once-filtered and unfiltered specimens. Although exactly comparable activities can not be presented, certain facts relating to the identity of the two factors are apparent.

Table 22

Effect of Normal Bovine Serum on the Prozone Growth-Inhibiting Action of Serum From a Brucella-Infected Cow

Serum No.	Age of serum[d] (days)	Normal bovine serum, ml.[a]						Bacterial control[b]
		Unheated			Heated[c]			
		0.5	0.25	0.1	0.5	0.25	0.1	
1	0	-						
	6	2+	2+	4+				
	10	1+	4+	4+				
	30	4+	4+	4+				5+
2	1	-	-	-	2+	3+	4+	
	19	1+	2+	4+				4+
	56	4+	5+					5+
3	4	-	3+	5+				
	41	2+	4+					4+
	71	4+	4+					5+
4	6	-	4+	5+	5+	5+	5+	5+
	28	5+	5+					4+
5	4	-	2+	4+				
6	1	2+	2+	-	3+	3+	3+	

[a]Normal serum added to a 1:100 test dilution of serum from a brucella-infected cow in culture medium (5 ml.). Test dilution also contains 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine. Incubation period, 72 hours.

[b]Control tubes of medium (5 ml.) contain a 1:100 dilution of serum from the brucella-infected cow, 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum, and 0.01 mg. sodium sulfadiazine.

[c]Serum heated at 56° C. for 30 minutes.

[d]Stored at 4° C.

- = no visible growth. + = degree of growth.

A relationship was noted between a prozone effect in the growth-inhibiting activity of normal serum 6 *per se* and a similar effect in its prozone-inhibiting activity. Of the six serums, only serum 6 (animal 5, table 13) showed a prozone in its growth-inhibiting action, and it was the only serum to evidence complete prozone-inhibiting action when present in a high dilution but not in a low dilution.

On the other hand, three normal bovine serums, less than a week old and capable of growth-inhibition at dilutions of 1:40 or greater during that time, failed to exhibit prozone-inhibition in a dilution of 1:20. This indicates either a modification of the growth-inhibiting activity when acting in the presence of specific antibody or an independence of the two factors.

b. Normal serum fractions. -- The ability of fractions from normal serum, in the presence of sodium sulfadiazine, to affect the prozone reaction of serum from a brucella-infected cow is demonstrated by the data in table 23. This property was determined by the 1-tube method using the amounts of serum fractions indicated. The preparation of the fractions is described elsewhere, and their growth-inhibiting activities are presented in table 10.

Examination of the data reveals that none of the 15-percent fractions evidenced any inhibitory effect on the prozone growth, and all the 20-percent fractions proved active in this respect. The 20-percent supernatant fraction of serum 5 inhibited prozone growth and that of serum 4 retarded but did not completely inhibit it. The 3+ growth in tubes containing 0.5 ml. of supernatants 1 and 2 might represent a slight activity but the significance is doubtful.

The foregoing results show that certain serum fractions, in the

Table 23

Effect of Normal Bovine Serum and Its Fractions on the Prozone Growth-Inhibiting Action of Serum From a Brucella-Infected Cow

Serum No.	Substance	Age of serum (days)	Normal bovine serum and fractions[a]		
			0.5 ml.	0.25 ml.	0.1 ml.
1	Serum, whole	5	2+	2+	4+
	Fraction A[d]		4+		
	Fraction B[d]		-		
	Fraction C		3+		
2	Serum, whole	6	-	4+	5+
	Fraction A[c]		5+	5+	5+
	Fraction B[d]		-	1+	3+
	Fraction C[b]		3+	4+	
4	Serum, whole	19	1+	2+	4+
	Fraction A[c]		6+	6+	6+
	Fraction B[d]		±		4+
	Fraction C[b]		2+	2+	4+
5	Serum, whole	4	-	3+	5+
	Fraction A[c]		5+	5+	5+
	Fraction B[d]		-	-	1+
	Fraction C[b]		-	2+	4+

A = fraction precipitated at 15-percent sodium sulfate concentration.

B = fraction precipitated at 20-percent sodium sulfate concentration after removal of A.

C = supernatant of fraction B.

[a]Normal serum or fraction added to a 1:100 dilution of serum from a brucella-infected cow in 5 ml. of culture medium which also contains 10^5 Br. abortus, 0.2 ml. of fresh, normal rabbit serum and 0.01 mg. sodium sulfadiazine. Incubation period, 72 hours. Bacterial controls as in Table 22.

[b]Concentrated to one-half the original serum volume.

[c]Concentrated to one-third the original serum volume.

[d]Concentrated to one-quarter the original serum volume.

- = no visible growth. + = degree of growth, turbidity.

presence of sodium sulfadiazine, were able to inhibit prozone growth. Whether any relationship exists between the growth-inhibiting activity per se and the prozone-inhibiting activity is not clear. The four 15-percent fractions showed a low growth-inhibiting activity at dilutions of 1:10 and no effect against the prozone growth. Although three of the four 20-percent fractions were capable of completely inhibiting growth in dilutions of 1:20, the fourth possessed no activity. However, all four samples caused prozone inhibition. Of the four supernatants, one had shown a definite and one a very slight growth-inhibition; only these two were effective against prozone growth.

c. Normal colostrum. -- Seven colostral wheys were tested for prozone-inhibiting action by the 1-tube method. The results presented in the last column of table 8 demonstrate that colostrum resembles normal serum in its ability to prevent or retard prozone growth in the presence of sodium sulfadiazine. The seven colostrums evidenced different degrees of prozone-inhibiting action which showed no apparent correlation with their independent growth-inhibiting action, in the presence of sodium sulfadiazine and complement, although both tests were done on identical colostral whey samples.

2. The effect of certain physical forces on the prozone-inhibiting action of serum

Since inconclusive, or contradictory, results concerning the correlation of growth-inhibiting and prozone-inhibiting activity had been obtained, the effect of physical forces on the prozone-inhibiting activity was investigated. Heating at 56° C. for 30 minutes, storage, and filtration had been shown to reduce the growth-inhibiting activity of normal bovine serum. The following data demonstrate a similar effect on

the prozone-inhibiting activity of bovine serum.

a. Effect of heat. -- Three normal serums were heated at 56° C. for 30 minutes and their effect on growth in the 1-tube prozone test was compared to that of unheated serum. Data in table 22 on serums 2, 4, and 6 show that the ability of normal serum to abolish prozone growth was destroyed, or reduced, at 56° C. for 30 minutes. Some residual activity remained in serum 2 after heating for while the 1:10 dilution of serum did not prevent growth it did retard it.

To determine whether heating altered the prozone-inhibiting activity of fractions from serum as it did whole serum, two active 20-percent fractions were heated at 56° C. for 30 minutes, and their prozone-inhibiting activity was compared with that of the unheated fractions. As with whole serum, heating destroyed the ability of the two fractions, in the presence of sodium sulfadiazine, to function against the prozone phenomenon in serum from a brucella-infected cow.

b. Effect of storage. -- Four serums were stored at 4° C. and tested at intervals by the 1-tube method. The data in table 22 concerning storage indicates that the prozone-inhibiting action disappeared within 1 or 2 months. Serums 1 and 4 showed no activity at 4 weeks. Serum 2 exhibited a reduced activity at 3 weeks and none at 8 weeks. Serum 3 remained somewhat active against the prozone reaction for 6 weeks but not for 10 weeks.

Data, not presented in this report, on the effect of storage at 4° C. on the ability of fractions from serum to prevent prozone growth indicate that this activity diminished with time and disappeared between 1 and 2 months. Serum fractions thus retained this property for approximately the same length of time as whole serum.

c. Effect of filtration. -- The reduction of the prozone reaction effected by two fresh, normal serums before filtration and after passage through seven Hormann D8 pads was compared in the presence of sodium sulfadiazine. Serum from the brucella-infected cow 991 was diluted 1:10 and thence through four twofold dilutions. To each tube were added 0.5 ml. of fresh or filtered normal serum and the growth-inhibition test reagents. After an incubation period of 72 hours the dilutions containing unfiltered serum showed reduced growth, represented by 1+ or 2+, as compared to a 5+ growth in the presence of serum filtered seven times. From these results it appears that the amount of substance capable of preventing prozone growth in serum from a brucella-infected cow, in the presence of sodium sulfadiazine, was reduced by filtration.

DISCUSSION

An examination of the *in vitro* growth-inhibiting activity of serum from calves after the ingestion of colostrum, as compared to that of adult animals, leads one to question the significance of normal growth-inhibiting activity as an index of resistance. At this time, when any passive protection conferred by colostrum should be at its maximum, the growth-inhibiting activity of serum from the calf is less than that from the adult animal. Furthermore, if a relationship exists between resistance and growth-inhibition, serum from the pregnant cow should exhibit a drop in activity for this is the period when the animal is most susceptible to brucellosis. However, data not presented in the report showed no significant variation in the growth-inhibition titer of serum from an animal taken at intervals before and during the pregnancy.

An unidentified factor which is responsible for the resistance of the calf to brucellosis may well be transmitted by colostrum. A complement-independent principle capable of brucella growth-inhibition in the presence of sodium sulfadiazine has been detected in seven out of eight colostrums although a similar mechanism has not been shown in the blood of calves or adult animals. It is well established that the concentration of specific antibodies in colostrum exceeds that of the maternal blood. Possibly the concentration of this principle in colostrum places it just within the range of our present means of detection. If the substance were selectively absorbed from the intestine of the calf, as the specific antibodies have been shown to be, subsequent dilution in the blood would reduce it beyond the limits of our

method although its concentration at that period might far exceed that of the adult animal. On the other hand, this factor may be peculiar to colostrum for the mammary gland synthesizes proteins not found in serum and even imparts its own characteristics to those which are closely related to serum proteins (10).

In this study the normal growth-inhibiting factor against Brucella has been demonstrated in the serum of calves prior to the ingestion of colostrum. Hansen and Phillips (30) recently reported that the serum of the newborn calf, before the ingestion of colostrum, contains small amounts of proteins immunologically similar to colostrum "immune" proteins. These were detected by precipitin methods using antiserums to purified pseudoglobulin from bovine colostrum. According to the present concept, the relative importance of colostrum to the newborn animal is correlated with the placental structure of the species. If this is the case, the proteins present in the serum of the calf at birth must be of such a nature as to pass the placental barrier and the normal growth-inhibiting antibody, in distinction to the specific, must be of this class.

Although the experiments described in this paper failed to clarify the significance of the natural brucella growth-inhibiting antibody, certain of its characteristics in the presence of sodium sulfadiazine have been made apparent.

The normal growth-inhibiting factor shows a marked lability at 56° C.; in some instances the heated serum was unable to inhibit growth in a dilution of 1:10 or greater, and in other instances it exhibited a less pronounced reduction in activity which was accompanied by prozone growth. These results are in contrast to those of Mackie and Finkelstein

who found the natural bactericidins generally stable at 55° C. Although concluding that bactericidins were not destroyed at this temperature, they noted that an inhibiting substance from unwashed organisms was unable in some cases to inactivate the bactericidin in unheated serum but inactivated it in heated serum. This difference in sensitivity of the bactericidin led them to suggest that some additional factor, possible thermolabile, was concerned in the bactericidal phenomenon. It is presumed that the increased sensitivity of the sulfadiazine-antibody-complement system accounts for the discrepancy between their results and ours. On the other hand, it may be due to differences in the individual bactericidins and organisms studied. The other natural complement-fixing antibodies which react with bacterial antigens are labile at 55° C., and it is not surprising to find the natural brucella growth-inhibiting factor in this class. Natural opsonins have long been recognized as thermolabile at 56° C. Ginsberg and Horsfall (31) found the component of normal serum which neutralized the infectivity of several viruses to be thermolabile at 56° C. Likewise, Chang (32) reported the spermicidal substance of fresh normal serums, including bovine, to be in this category. However, in the latter investigation the lability does not appear to be distinguished, with certainty, from a complement requirement of the system.

From studies of the effect of heat on specific antiserums, reviewed and extended by Follensby and Hooker (33), it would appear that the reduced activity and prozone growth of heated normal serum might result from a similar mechanism taking place at lower temperatures. Shibley (34) showed that heating induced prozone agglutination of antiserums, and he confirmed the finding of Jones (35) that heated antiserums

inhibited the action of unheated antiserums. Van der Scheer et al. (36) and Hardt et al. (37) have demonstrated electrophoretically that heating of normal serum at 56° or 65° C. causes the formation of a colloidal complex at the expense of various serum components. In examining the effect of heat on the formation of protein complexes, Kleczkowski (38), (39) and Bawden and Kleczkowski (40) demonstrated that antiserums which were partly denatured by heat behaved like mixtures of particles with different properties, all able to combine with antigen but only some able to cause precipitation. The mixed complex inhibited precipitation by the regular antibody. Jennings and Smith (41) showed that heating of antipneumococcal antiserum with non-specific protein reduced its precipitability and the concomitant formation of a new component was confirmed electrophoretically by Krejci et al. (42).

While the formation of a new component could account for the reduced activity and prozone reaction of heated normal serum, it fails to explain the difference in sensitivity of the specific and normal growth-inhibitors to heat. In view of the demonstration by Hardt et al. (37) of the protective action of certain sugars against heat denaturation of serum proteins, it seems possible that the relatively high resistance of colostrum to heat and storage, as compared to serum, may be a function of its high sugar concentration.

It has been shown that storage of serums and serum fractions at 4° C. also resulted in a reduction, and in some cases disappearance, of their growth-inhibiting activity in the presence of sodium sulfadiazine. As the effect of heating and storage on the activity of serum is similar, it may result from the same reaction proceeding at different rates. It would be of interest to attempt to confirm this

electrophoretically and to correlate the formation of a new component with the reduction in growth-inhibiting activity.

In these experiments filtration of normal serum through asbestos pads invariably resulted in a slight reduction of the growth-inhibiting activity. Huddleson et al. obtained quite the opposite results as filtration increased the bactericidal activity of normal plasma acting without the presence of sodium sulfadiazine. They failed in attempts to elute any inhibitor of the reaction from the filter. The reduction in growth-inhibiting activity demonstrated in this study may have been due either to the retention of material on the filter or to the addition of ions from the asbestos pad to the filtrate. The presence of the magnesium ion and other cations in the filtrate after passage through asbestos filters is well known. Mayer and his associates (43) have demonstrated that the magnesium ion has a stimulatory or inhibitory effect on complement-fixation, the effect depending on its concentration and ratio to other ions such as calcium. Possibly the magnesium ion concentration following filtration favors the bactericidal action of citrated plasma and retards that of serum in the presence of sodium sulfadiazine. Successive filtration through seven asbestos filter pads progressively reduced the activity of the normal sulfadiazine-antibody-complement system. However, each filtration reduced a smaller proportion of the residual activity. Whether this represents the removal of one of two growth-inhibiting factors in the serum or approach to maximum inhibitory effect of ions added to the filtrate is not known.

The sulfadiazine-antibody-complement system proved to be very sensitive for detecting normal growth-inhibiting action against Brucella. For example, normal bovine serum in an average dilution of 1:80 and in

the presence of sodium sulfadiazine and complement inhibited the growth of 10^5 Br. abortus organisms. The method itself is subject to errors inherent in the use of biological reagents. While fresh, normal rabbit serum was not able to inhibit growth in the concentration at which it was used as a reagent, it exhibited a slight but variable effect on the growth-inhibiting action of normal bovine serum. Although evidence of the additive effect did not extend beyond one twofold dilution, comparative results were obtained from tests conducted with the same rabbit serum with the one exception noted. A more significant error was the variation encountered in the effect of the normal growth-inhibitor on dissociated growth phases of Br. abortus which were not discernible by gross examination of the culture. This can be avoided only by placing proper emphasis on the cultural phase of the organism used for the bacterial suspension.

No explanation can be offered for the discrepancy between our results and those of Irwin and his associates who found normal rabbit serum inadequate as a source of complement for detecting bovine serum bactericidins against Brucella. Possibly sodium sulfadiazine is capable of augmenting some deficiency in normal rabbit serum, or growth-inhibiting action in the sulfadiazine-antibody-complement system may be the manifestation of a different bactericidal mechanism. The latter does not seem likely to the author. Results not presented here indicate that the transient growth-inhibition titer evident without the presence of sodium sulfadiazine and in the early hours of incubation coincides with that observed far more distinctly after 72 hours of incubation in the presence of sodium sulfadiazine.

SUMMARY

Normal bovine serum, in the presence of sodium sulfadiazine and added complement, was capable of inhibiting the growth of 10^5 Br. abortus for 72 hours when in an average dilution of 1:80. The growth-inhibiting factor resembled other natural complement-fixing antibodies in being thermolabile at 56° C., as compared with the specific antibody which was stable at this temperature. Both storage at 4° C. and filtration reduced the growth-inhibiting action of normal bovine serum.

A brucella growth-inhibiting factor was demonstrated in the serum of newborn calves before the ingestion of colostrum. Serum taken from calves 48 hours after the ingestion of colostrum inhibited growth in higher dilutions than serum taken from calves at birth, but its activity did not exceed that of the adult animal.

Colostral whey was found to possess two growth-inhibiting factors, one which did not require complement and a second dependent on the presence of complement to inhibit growth of Br. abortus in the presence of sodium sulfadiazine. The effect of heat, storage, and filtration on the colostral growth-inhibiting activity was less than on the corresponding serum factor.

Fractions of serum from one brucella-infected cow and five normal cows were separated by precipitation with different concentrations of sodium sulfate and examined with respect to growth-inhibiting activity and electrophoretic components. The growth-inhibiting factor of the specific antiserum was insoluble in 15 percent sodium sulfate; the major part of the factor in three of the five normal serums was soluble in

15 percent but insoluble in 20 percent sodium sulfate. A correlation was noted between the γ-globulin content and the growth-inhibiting activity of fractions from specific antiserum. No correlation existed between the γ-globulin content and the activity of fractions from normal serum.

In the presence of sodium sulfadiazine fresh, normal bovine serum and colostrum were able to inhibit prozone growth in serum from a brucella-infected cow. The identity of this factor with the growth-inhibiting factor was examined.

Fresh, normal rabbit serum served as an adequate source of complement for the brucella growth-inhibiting action of normal bovine serum in the presence of sodium sulfadiazine.

It was demonstrated that the use of different, but closely related, growth phases of Br. abortus influenced the results of the sulfadiazine-antibody-complement titrations. This constitutes a potential source of error in comparative studies.

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